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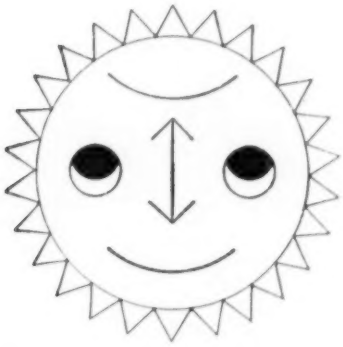

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Newcastle Disease Immunization Studies. I. Viability of Newcastle Disease Virus Administered as a Vaccine in the Drinking Water

R. W. WINTERFIELD, D.V.M., and E. H. SEADALE, M.S.

Amherst, Massachusetts

LITERATURE CITED

Mass immunization techniques for poultry have been reported by Hitchner and Reising,[1,2] Luginbuhl *et al.*,[3] Markham *et al.*,[4] and others.[5,6] These procedures of administration, including the water route, have numerous advantages and have met with popular appeal among commercial poultrymen. Many of the limitations and disadvantages of these programs, however, have not been fully ascertained or described.

The viability of Newcastle disease virus (NDV) under varied conditions has been studied by Olesiuk[7] and also by Wooding.[8] Beaudette[9,10] has summarized survival studies on several NDV strains. Cunningham[11,12] and others[13] have investigated the effects of chemical agents on NDV. Luginbuhl[3] reported that infected allantoic fluid added to the drinking water at a rate of 0.5 ml. per gallon, giving a final concentration of 10^6 of B1 strain of NDV, remained viable after 28 hours. That NDV will survive for extended periods under adverse conditions has been well established. The effects of various field environmental factors on titers of water-administered NDV, however, have not been clearly defined.

It was the purpose of this investigation to determine the favorable or unfavorable influence which some of these conditions may have on vaccine water titers.

MATERIALS AND METHODS

The B1 strain[14] of NDV used in all trials was obtained from four different sources: Vaccine "J" was a dried virus from a commercial source, available in 500-dose vials with 15 ml. of diluent supplied with each vial. Vaccine "K," also commercially available, represented a glycerinated virus. This vaccine was received in a 1,000-dose, 60-ml. quantity and was accompanied by a dried nutrient stabilizer. Vaccine "M," in the form of amnioallantoic fluid, was obtained in this laboratory from B1-NDV seed stock. Vaccine "L" was lyophilized in 3-ml. quantities from a 1:1 mixture of 10 per cent skim milk and amnioallantoic fluid. Each serial lot or pool of vaccine was titrated at least once prior to its use.

Water samples from 11 sources were obtained for study and chemically analyzed[*] (table 1). Where data are incomplete, insufficient water was available for complete analysis. Sample 1, used as a control in each trial, was single-distilled sterile water. In two trials, NaCl was added to the No. 1 samples to give a final concentration of 0.85 per cent. With the exception of sample 8, samples 2 through 11 represented water from wells or taps on Massachusetts poultry farms and were used without regard to sterility. Sample 8 came from central Ohio and represented an alkaline water. Sample 2, considered hard for New England water from natural sources, was also high in total solids, volatile matter, and chlorides. Except for a small amount of unfiltered iron (Fe), sample 3 presented no extreme in its analysis and was included merely as a field sample. Sample 4 demonstrated relatively heavy contamination by various protozoa, algae, and other microscopic agents. The excessive amount of unfiltered iron in this sample was caused by water standing for one week in a galvanized iron poultry water trough. The No. 4 water was normally low in soluble iron. The presence or absence of rust when sample 4 was used is indicated in the accompanying tables. Numbers 5 and 6 were field samples, with sample 6 having fluorides added. Sample 7 came from a farm with a mountain spring as its water source. Soil in this area was high in calcium, which was reflected in water hardness. As previously men-

Contribution No. 1005 of the Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst.

[*] Analyses of water samples were made by Mr. E. A. Snow, Water and Sewage Laboratory, Department of Public Health, Amherst, Mass.

tioned, sample 8 was a hard, alkaline, midwestern water sample. Numbers 9, 10, and 11 were field samples with no peculiarities in their analyses.

Three water samples were included in each trial where temperature effects were studied. In all others, four samples were used in each trial. Thirty minutes before each trial the water samples, contained in 1,000-ml. Ehrlenmeyer flasks, were placed in a water bath and brought to the desired temperature to be maintained during the trial. Levels of water in the flasks and bath were approximately the same in all trials, as were the exposed surface areas of the water samples.

Four trials were conducted at temperatures of 24 C., 31 C., and 38 C. These temperature ranges bracket the normal and higher temperatures found in brooding chicks from 1 day to 4 weeks of age. The upper limit of 38 C. is occasionally present in the summer months. All other trials were carried out at 31 C., as this more closely approximates the floor temperatures near the outside of the brooder hover when the chicks are 1 to 10 days of age.

When contamination was the subject of study, the following materials, and quantities of each per liter, were added: pine shavings litter, 5 Gm.; cane litter, 5 Gm.; commercial starting mash (without a coccidiostat but containing a broad spectrum antibiotic), 5 Gm.; and fresh chicken feces, 25 Gm. Field observations indicate these types of materials as common contaminants of poultry waterers. Quantities used, however, were arbitrary but apparently within the limits of reality.

As many eastern poultry farms have a city source of water, it was desirable to determine the effects of chlorination. Chlorine gas was added to the test water from a stock solution of water containing 375 parts per million (p.p.m.) The residual chlorine content of the test water was adjusted to a final concentration of 0.1 p.p.m. by means of an orthotolidine indicator.[16] This level of residual chlorine is often used in city water supplies.

In trials where the commercial dried virus (J) was used, the material was reconstituted with 15 ml. of diluent supplied. Lyophilized virus (L) from this laboratory was reconstituted with 15 ml. of sterile, distilled water. Each vaccine was added to the test water sample in the flasks in the ratio of 1 ml. to 315.3 ml. of water. The stabilizer supplied with vaccine K, when used, was added in a 0.41-Gm. amount to 30 ml. of the test water sample with the virus as a premixture and then added to give the original volume. A stabilizer consisting of two-thirds purified gelatin and one-third dried skim milk was used in one trial with vaccine L in the ratio of 1.3 Gm. to 315.3 ml. of water. No attempt was made to demonstrate the best types and amounts of stabilizing materials.

All virus-containing water samples were sampled in 0.5-ml. amounts as near the surface as possible at intervals of five minutes, one hour, two hours, four hours, eight hours, and 24 hours, post-inoculation. In a few cases, the sampling was extended beyond this limit. It was felt that more frequent quantitative viral determinations would be desirable in the earlier hours of the trials, because fowl can be water-fasted prior to vaccination and the dosage administered in a shorter time. Serial tenfold dilutions were made of the inoculum in extract broth containing 10,000 units of crystalline penicillin G and 25 mg. of dihydrostreptomycin per milliliter. An incubation period of 15 minutes at 25 C. followed. In tests involving feed, feces, and litter, the antibiotic levels were doubled and the incubation period extended to 20 minutes for all samples in the trial. After incubation, each dilution was inoculated into four 10-day-old chick embryos via the allantoic sac. An inoculum of 0.1 ml. per egg was delivered with a 1-ml. tuberculin syringe and a ⅝-in., 25-gauge needle. A small quantity of each dilution was placed in thioglycolate medium for sterility check. The eggs were then returned to the incubator and candled once daily thereafter for five days. The 24-hour mortalities were discarded as nonspecific.

TABLE 1—Analyses of Samples of Water Used

Analysis in parts per million	Water samples (No.)										
	1	2	3	4	5	6	7	8	9	10	11
Color	0.0	4.0	27.0	....	....	2.0	....	....	12.0	3.0	3.0
Silica	0.0	6.0	....	4.0	7.0	....	5.0	5.0	12.0	6.5	....
Total solids	2.2	2,780.0	105.0	322.0	56.0	88.0	92.0	319.0	58.0	65.0	26.0
Volatile matter	1.8	2,636.0	23.0	73.0	15.0	20.0	26.0	48.0	17.0	12.0	4.0
Nitrate	0.01	0.03	1.1	0.03	0.60	0.10	0.05	1.1	0.10	0.4	0.05
Nitrite	0.000	0.000	0.014	Trace	....	0.002	0.000	0.010	0.000	0.000	0.000
Chlorides	0.2	1,420.0	12.8	5.2	4.0	5.2	1.4	15.0	2.2	5.0	2.6
Hardness	2.0	524.0	64.0	18.0	20.0	40.0	74.0	106.0	16.0	28.0	12.0
Alkali	4.0	16.0	26.0	8.0	16.0	26.0	68.0	8.0	14.0	36.0	10.0
pH	6.5	7.4	5.9	6.5	7.5	6.5	7.2	9.2	6.5	6.5	6.5
Sulphate	....*	37.2	18.1	8.6	16.1	7.8	1.9	160.0	7.4	16.4	18.1
Calcium	0.0	191.0	32.0	6.0	12.0	26.0	48.0	61.0	8.0	22.0	10.0
Sodium	....*	1,200.0	18.0	....	32.0	48.0	1.0	56.0	36.0	....	24.0
Potassium	....*	1.7	10.0	....	0.2	5.0	0.0	0.1	4.0	....	0.1
Magnesium	....*	....	32.0bd	12.0bd	8.0bd	14.0bd	26.0bd	45.0bd	8.0bd	6.0bd	2.0bd
Iron	0.05	....	5.2	65.0† 0.20‡	0.17	.....	0.18	0.08	.....	.....	.....
Fluoride	....	....	....	....	....	0.6±0.2	....	....	....	....	....

* Trace; † unfiltered; ‡ filtered; bd—determined by difference.

TABLE 2—Effect of Temperature on Vaccines

Trial (No.)	Water sample	Water temp. (C.)	Vaccine	Vaccine titer	Log embryo infective doses$_{50}$/ml. water						
					5 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1A	1	24	J Stabilizer —	7.5	5.5	4.7	4.5	4.7	4.7	4.5	3.7
B	1	31		7.5	5.3	4.3	4.7	4.5	4.2	4.5	2.5
C	1	38		7.5	5.5	4.5	4.3	3.7	3.5	3.3	0.0
2A	1	24	J Stabilizer —	7.5	5.2	5.2	4.7	4.5	4.5	4.5	4.7
B	1	31		7.5	5.5	5.2	4.5	4.5	4.3	3.7	3.3
C	1	38		7.5	5.3	5.2	4.7	4.0	3.7	3.0	0.0
3A	1	24	K Stabilizer +	7.6	5.5	5.5	5.3	5.0	5.3	5.6	5.0
B	1	31		7.6	5.3	5.2	4.5	5.0	5.5	5.1	4.3
C	1	38		7.6	5.5	5.6	5.0	4.3	5.0	5.5	4.6
4A	1	24	K Stabilizer +	7.6	6.0	5.5	5.5	5.0	5.3	5.0	5.5
B	1	31		7.6	5.7	5.2	5.5	5.7	5.3	5.5	3.7
C	1	38		7.6	6.0	5.5	5.5	5.7	5.0	5.2	5.0

— Absence of stabilizer; + presence of stabilizer.

Titration end points were calculated by the method of Reed and Muench[16] from mortalities, and also by the hemagglutinative activity of the allantoic fluid in live embryos at the end of the fifth day. Log embryo infective doses$_{50}$ (e.i.d.$_{50}$) per milliliter of water were determined.

Determinations of pH were accomplished on all water samples at periodic intervals during each experiment with a model "H" Beckman pH meter.

RESULTS

Tables 2 through 6 indicate the resulting titrations of Bl strain of NDV from the water samples identified by numbers in table 1. Vaccines are designated by their assigned letters. The presence (+) or absence (—) of a stabilizer, as well as vaccine titers, is also expressed.

Temperatures.—The data presented (trials 1 and 2, table 2) for vaccine J indicate only a slight, gradual decline in titer beyond the initial titer at five minutes with 24 C. and 31 C. After four hours, the drop in titer was more pronounced at 38 C. than at lower temperatures. No virus was detected at 24 hours in either trial. Table 2 indicates that in trials 3 and 4 vaccine K, with stabilizer, demonstrated little inclination to drop in titer, even at 38 C. It is apparent that high titers of Bl-NDV can be maintained in water at normal brooding temperatures for at least 24 hours.

Stabilizers.—Table 3 clearly indicates that higher titers of NDV were maintained when a stabilizer was used. Vaccine K with stabilizer maintained higher titers in control and field water samples (1, A and C) than sustained when the stabilizer was not included (1, B and D). When vaccine

TABLE 3—Effect on Vaccine of Stabilizers at 31 C.

Trial (No.)	Water sample	Vaccine	Vaccine titer	Log embryo infective doses$_{50}$/ml. water						
				5 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1A	1	K Stabilizer +	7.6	5.7	6.0	5.5	5.7	6.0	5.7	5.3
B	1	K Stabilizer —	7.6	5.3	5.0	5.2	5.0	5.0	4.3	4.0
C	5	K Stabilizer +	7.6	5.3	6.0	5.3	5.0	6.0	5.2	3.1
D	5	K Stabilizer —	7.6	5.0	5.5	5.0	4.1	3.3	2.5	1.7
2A	1	J Stabilizer +	7.5	5.3	5.0	5.5	5.2	5.2	5.0	5.5
B	1	J Stabilizer —	7.5	5.0	5.5	4.7	4.7	4.5	4.3	4.0
C	5	J Stabilizer +	7.5	5.5	5.6	4.8	5.3	5.3	5.3	5.0
D	5	J Stabilizer —	7.5	5.6	4.7	4.7	4.3	4.3	4.2	0.0
3A	1	L Stabilizer +	8.6	6.7	6.5	7.7	6.5	6.7	6.2	5.7
B	1	L Stabilizer —	8.6	6.3	7.0	6.7	6.0	6.0	5.5	4.7
C	11	L Stabilizer +	8.6	6.7	6.7	6.5	4.6	6.6	4.0	3.5
D	11	L Stabilizer —	8.6	6.5	5.5	5.3	4.5	3.6	3.5	0.0

— Absence of stabilizer; + presence of stabilizer.

J was tested with the stabilizer supplied with vaccine K, similar results were observed in both trials 1 and 2. When used with the gelatin-skim milk preparation, vaccine L gave somewhat higher titers in control and field samples (3, A and C) than when the preparation was absent (3, B and D).

Although the stabilizing materials occasionally settled or precipitated out of solution, it appeared to have no effect on titer. In several trials, attempts to sterilize stabilizer-containing solutions with a Swinny filter resulted in the removal of most of the NDV particles.

Chlorine in Presence or Absence of Sabilizers.—That chlorinated water is markedly deleterious to NDV is seen in table 4 (trials 5, C; 6, D; 7, B; and 8, B). No significant effect was observed, however, when glycerinated vaccine K was used with its stabilizer (4, C). In contrast, vaccine J was quickly destroyed (5, C). In trial 6, the viability of vaccine K remained satisfactory, while vaccine J was again rapidly destroyed. When the stabilizer of K was used with J (7, C), however, the viability remained high. In contrast, use of vaccine K without its stabilizer resulted in rapid destruction of the virus (8, B). This indicated that the stabilizer and not the glycerine was responsible for the protective influence.

Feed and Fecal Contamination.—The harmful effects of feed and fecal contamination (singly or together) are shown by the data in table 4 (trials 1, B, C, and D; 2, D; 3, D; 4, D; and 5, D). Although decreases in titers were more prevalent in the later hours, a marked decrease with feed contamination was present in trial 1 after one hour. Similar results were obtained with combined feed and fecal contamination. Bacterial growth was excessive in these samples, particularly beyond the fifth or sixth hour; higher antibiotic levels

TABLE 4—Effect on Vaccine of Feed, Fecal, and Rust Contamination, Chlorination (with and without Stabilizers), and Alkaline Water at 31 C.

Trial (No.)	Water sample	Vaccine	Vaccine titer	Log embryo infective dose$_{50}$/ml. water						
				5 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1A	1	L Stabilizer —	8.6	6.5	6.5	6.5	5.8	5.7	6.0	4.5
B	1 + Feed		8.6	6.7	3.8	2.7	0.0	0.0	0.0	0.0
C	1 + Feces		8.6	6.2	6.5	5.2	5.5	4.8	4.7	1.7
D	1 + Feces and Feed		8.6	5.7	3.7	2.7	0.0	0.0	0.0	0.0
2A	1	J Stabilizer —	7.5	5.2	5.0	5.3	4.7	4.3	4.0	3.3
B	4		7.5	5.0	5.0	5.3	3.5	4.0	3.3	0.0
C	4 + Excessive Rust		7.5	5.0	4.5	5.3	3.7	3.3	3.5	1.7
D	4 + Feces		7.5	5.3	5.0	4.5	3.7	3.5	2.8	0.0
3A	1	K Stabilizer +	7.6	5.5	5.5	5.5	5.0	5.3	5.5	4.5
B	4		7.6	5.5	4.8	5.5	4.3	4.5	4.5	2.3
C	4 + Excessive Rust		7.6	5.5	5.5	5.6	4.5	4.5	5.0	4.6
D	4 + Feces		7.6	5.5	6.0	5.3	5.1	4.5	5.2	0.0
4A	1	K Stabilizer +	7.9	6.0	6.3	5.7	6.0	6.3	5.3	5.3
B	7		7.9	5.7	5.7	6.0	6.0	6.3	5.2	5.0
C	7 + Chlorine		7.9	5.7	5.5	6.0	5.5	5.7	6.3	3.5
D	7 + Feed		7.9	6.3	6.0	6.0	5.5	5.3	5.3	2.0
5A	1	J Stabilizer —	7.7	5.5	5.5	5.0	4.5	5.3	4.5	3.5
B	7		7.7	5.7	5.3	5.7	5.5	4.5	4.6	3.3
C	7 + Chlorine		7.7	2.7	0.0	0.0	0.0	0.0	0.0	0.0
D	7 + Feed		7.7	5.7	5.3	5.0	4.0	4.3	3.5	1.6
6A	1	K Stabilizer +	8.9	6.3	5.7	6.0	5.7	5.7	6.3	4.5
B	1 + Chlorine		8.9	6.0	5.5	5.7	5.5	5.0	4.7	5.0
C	1	J Stabilizer —	7.7	5.3	5.3	5.7	5.3	5.0	4.2	4.3
D	1 + Chlorine		7.7	5.0	5.2	0.0	0.0	0.0	0.0	0.0
7A	1	J Stabilizer —	7.7	5.3	6.0	5.0	5.3	5.2	5.0	3.3
B	1 + Chlorine		7.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C	1 + Chlorine	J Stabilizer +	7.7	6.0	4.5	4.7	5.3	5.5	5.3	4.3
D	8	J Stabilizer —	7.7	5.0	5.3	4.7	4.6	5.5	5.0	5.3
8A	1	K Stabilizer +	8.9	5.7	6.3	6.3	6.3	6.0	5.6	5.2
B	1 + Chlorine	K Stabilizer —	8.9	2.5	0.0	0.0	0.0	0.0	0.0	0.0
C	1 + Chlorine	K	8.9	5.7	5.3	6.3	5.7	5.2	6.0	5.5
D	8	Stabilizer +	8.9	5.5	5.5	5.5	5.7	5.2	4.5	5.0

— Absence of stabilizer; + presence of stabilizer.

TABLE 5—Effect on Vaccine of Litter Contamination and a Fluoridated Water at 31 C.

Trial (No.)	Water sample	Vaccine	Vaccine titer	Log embryo infective dose$_{50}$ ml. water						
				5 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1A	1	K	7.9	5.7	5.5	5.5	5.7	5.8	5.7	6.0
B	6		7.9	5.7	5.5	4.7	6.0	6.0	6.0	5.6
C	6 + Wood shavings	Stabilizer +	7.9	5.7	5.5	5.3	5.3	5.7	5.0	0.0
D	6 + Cane litter		7.9	6.3	5.3	4.3	5.1	5.3	4.0	4.5
2A	1	J	7.5	5.5	4.6	4.8	4.3	4.7	4.3	3.3
B	6		7.5	5.5	5.0	4.5	4.2	3.7	4.0	0.0
C	6 + Wood shavings	Stabilizer −	7.5	5.5	3.5	2.8	0.0	0.0	0.0	0.0
D	6 + Cane litter		7.5	5.0	5.0	4.7	3.7	2.7	1.7	0.0

− Absence of stabilizer; + presence of stabilizer.

in the broth diluent were needed, therefore. Bacterial metabolic end products presumably exerted an effect on viability. This observation is somewhat similar to that made by Bankowski [17] in the propagation of NDV *in vitro*.

Rust or Iron.—The presence of excessive iron in water from a rusty water trough had no harmful effect on NDV (2, C and 3, C, table 4). In fact, titers were higher at the end of 24 hours in the water containing iron than in a similar noncontaminated water sample. This suggested the possibility of a stabilizing effect by the metal.

Variations in pH.—Ranges in pH of 4.4 to 9.2 had little effect on NDV titers. Water sample 8, although alkaline, maintained satisfactory viability for 24 hours (7, D and 8, D, table 4). In other trials (table 4, 3, C; table 6, 2, A, B, and C) as the water tended toward acidity, titers were not greatly affected. Fluctuations in pH were noted in some water samples during an experiment. Atmospheric conditions, bacterial growth, or other factors may have been influential in these changes. Previous observations by other investigators [18] indicate most animal viruses have optimum stabilities about pH 7, with ranges varying widely about this point.

Litter Contamination and Fluoridated Water.—Data in table 5 reveal the harmful effect of wood shavings as a contaminant of water (trials 1, C and 2, C). Where glycerinated virus and stabilizer were used, this effect was not apparent until after the eighth hour (1, C). However, the effects were seen as early as the first hour with vaccine J (2, C). It was also evident that cane litter contamination had some detrimental effect on titers after the fourth hour (1, D and 2, D).

When vaccine K was used with its stabilizer, titers remained as high in the fluoridated water as in the control. With vaccine J the titers remained about the same as the control for eight hours but dropped to zero by the twenty-fourth hour. It is impossible to state whether fluoridation or some other factor was responsible for this decline. It is evident that the glycerinated vaccine and stabilizer, however, were able to prevent this decline.

TABLE 6—Effect on Vaccine of Saline, Hard, and Randomly Selected Field Water Samples at 31 C.

Trial (No.)	Water sample	Vaccine	Vaccine titer	Log embryo infective dose$_{50}$ ml. water						
				5 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1A	1	J	7.5	5.7	5.5	5.0	5.3	4.7	4.5	3.0
B	1 + .85 NaCl		7.5	5.3	4.8	4.7	4.3	4.5	3.5	3.3
C	2	Stabilizer −	7.5	4.7	5.0	5.0	5.3	4.7	4.0	3.0
D	3		7.5	5.3	5.2	5.3	4.7	4.3	4.3	4.5
2A	1	K	7.6	5.5	5.7	5.5	6.0	6.3	6.0	4.9
B	1 + .85 NaCl		7.6	5.5	5.7	5.7	6.0	5.5	5.3	4.2
C	2	Stabilizer +	7.6	5.5	5.5	5.3	5.5	4.5	4.3	2.0
D	3		7.6	6.3	5.5	5.7	5.5	5.5	4.7	5.2
3A	1	L	8.6	>6.5	>6.5	>6.5	6.0	5.5	5.3	3.0
B	9		8.6	>6.5	>6.5	>6.5	6.0	6.3	6.5	5.7
C	10	Stabilizer −	8.6	>6.5	6.3	6.0	5.3	4.5	4.2	3.7
D	11		8.6	>6.5	6.3	6.0	4.5	3.3	3.0	0.0
4A	1	M	9.5	>7.5	>7.5	>7.5	>7.5	>7.5	>7.5	6.3
B	9		9.5	>7.5	>7.5	>7.5	>7.5	>7.5	7.0	>7.5
C	10	Stabilizer −	9.5	>7.5	7.3	6.6	7.3	5.5	6.0	4.3
D	11		9.5	>7.5	6.6	>7.5	6.5	6.6	6.6	3.0

− Absence of stabilizer; + presence of stabilizer.

Saline and Hard Waters.—Saline water was not found particularly harmful to NDV (table 6). Water sample 2, which was high in total solids, volatile matter, chlorides, and hardness, produced no harmful effect in trial 1. After four hours, only a slight depressing effect on titer was exerted in trial 2.

Randomly Selected Field Water Samples.—Of the field water samples in trials 3 and 4 (table 6), No. 9 and No. 10 had no effect on titers of vaccine L. Sample 11, on the other hand, decreased progressively to zero in 24 hours. With vaccine M, the decrease in virus titer was less noticeable because of its higher initial titer. Inasmuch as most waters are of complex chemical composition, it is difficult to state why some have adverse effects on viability and some do not. However, the viability of NDV was usually less affected in sterile distilled water than in field water samples.

Maximum Survival Time.—Although most of the trials were confined to a 24-hour period, a few titers were determined beyond this time: table 4, trial 3 A, 28 hours, $10^{-4.6}$; 32 hours, $10^{-5.5}$; 48 hours, $10^{-6.0}$; table 5, trial 1 B, 28 hours, $10^{-5.5}$; 32 hours, $10^{-4.7}$; 48 hours, $10^{-6.0}$; table 5, trial 2 A, 28 hours, $10^{-2.5}$; 32 hours, $10^{-2.9}$.

DISCUSSION

According to the experimental data, environmental factors may favorably or unfavorably influence water-administered ND vaccine. The significance of these results can not be understood, however, until dosage requirements and time necessary to administer the dosage are determined. Data[19] from preliminary work suggest that satisfactory immunity results from administering B1 strain of NDV in a two-hour period, after a four-hour water-fasting period. Results were similar to those obtained when the NDV dosage was administered for more than eight or 24 hours. If a satisfactory antigenic response can be obtained from administering the vaccine in a relatively short time, many of the undesirable effects determined in this study can be avoided. The actual dosage intake for each chick is more closely related to the titer of virus in the water than it is to the volume of water consumed.[19] With a high virus titer, a large dosage intake can be obtained in a comparatively short vaccine-exposure time.

Stabilizers demonstrate their greatest effect in maintaining higher titers for extended periods (more than 6 or 8 hours). Because of varied field conditions, it would seem desirable to use stabilizers in vaccine solutions even when administered for short periods. Although no attempt was made to determine the best types of stabilizing materials, it is well known that most viruses are more stable in mediums of complex organic composition.[18, 20] The use of salt-free purified gelatin, beef extract, blood serum, and dried milk is common practice in virus preservation. Viral activity may be protected against inactivating agents by foreign proteins acting as competition mechanisms. Prevention of surface denaturation has also been suggested.[18]

SUMMARY

1) Newcastle disease virus (B1 strain), under simulated field conditions of water administration, maintained its viability in some trials for as long as 32 hours. In other trials, shorter survival times were obtained depending upon environmental conditions.

2) Viability of the vaccine virus was adversely affected by high temperatures, chlorination, litter, feed and fecal contamination, a fluoridated water, and nonspecific factors (undetermined) in certain waters. Most adverse effects were not as pronounced during the first four to six hours as they were beyond this time; a higher dosage intake of Newcastle disease virus (NDV) during this early period, therefore, would avoid marked decreases in titers.

3) Essentially no effect on viability resulted from hard water, saline water, rust or iron contamination, and wide variations in pH.

4) Organic or animal-protein stabilizing materials, as well as a commercial stabilizer, were of definite value in enhancing NDV viability under adverse conditions. Most of these conditions were amenable to the use of stabilizers provided the initial NDV titers were satisfactory.

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The Etiology of an Arthritic Disease of Chickens

M. S. COVER, V.M.D., Ph.D.; J. N. GELETA, D.M.V.;
E. F. WALLER, D.V.M., M.S.

Newark, Delaware

PREVIOUS reports of arthritis in poultry have indicated various bacteria as etiological agents.

In 1931, Hole[1] stated that *Staphylococcus aureus* was responsible for an arthritis and periarthritis in pheasants, and Thorp *et al.*[2] reported an unusual form of fowl cholera in which arthritis was a prominent sign. Beaudette[3] in 1936 cited a case of arthritis in a chick caused by *Salmonella pullorum*. Recently Wills[4] and Olson *et al.*[5] reported the isolation from chickens of an agent which appeared unrelated to the cases previously reported. An agent similar to that discussed by the latter workers is the subject of this report.

It appears that an infectious arthritic disease is now present in chickens in the Delmarva area and may be increasing in importance. The disease has been observed only in young growing birds 4 to 12 weeks of age. Morbidity has been variable and reported to be as high as 30 per cent. The mortality has been low, about 2 per cent. However, the economic importance of the disease can be measured primarily in the number of culls and crippled birds which result.

The affected birds become weak, listless, and tend to assume a sitting position. They show lameness, the comb and wattles become pale and shrunken, a greenish diarrhea is almost a constant feature, and gross enlargement of the hock joints, footpads, wing joints, and keel bursae are usually seen.

Necropsies revealed the presence of tenacious, yellow to grey pus in the involved joint bursae and in many of the tendovaginal sheaths. The spleens were enlarged and the swollen livers had a definite green discoloration.

Careful dissection of the affected limbs showed that the flexor tendon sheaths were severely affected from a point just proximal to the hock to the area of the footpads. The synovial structures were thickened and contain a viscid, yellowish exudate which tended to become thicker in advance cases.

There has been no evidence to indicate that this disease has any clinical or etiological association with hemorrhagic disease or chronic respiratory disease. Arthritis may coexist with either of these diseases, however.

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EXPERIMENTAL DATA

All isolations of the agent were made from typically affected chickens which upon necropsy and laboratory tests showed no evidence of any complicating diseases. Exudate from the affected joints and tendon sheaths was withdrawn aseptically by means of a hypodermic syringe. This material was used as an inoculum for passage in embryonating chicken eggs after diluting it 1:5 with sterile physiological saline or nutrient broth. In some cases, additional inoculums were prepared by grinding liver or spleen tissue, or both, with sterile sand and nutrient broth.

Several lots of typically affected birds were necropsied in this laboratory during the spring of 1954. At that time, no effort was made to identify a possible etiological agent other than making routine bacteriological cultures from the joint exudate, liver, and spleen on nutrient agar, nutrient broth, and blood agar. None yielded any bacterial growth. A special change occurred on the blood agar medium inoculated with liver tissue. All of the cultures showed a faint zone of hemolysis along the line of inoculation. No colonies became visible along this line. The result of inoculation of suitable artificial medium (PPLO agar Difco) for the isolation of pleuropneumonia-like organisms (PPLO) was always negative.

Our first experimental work was done with chickens presented at the laboratory on May 16, 1954. An inoculum of liver and spleen tissue was prepared by grinding the tissue in a mortar with sand and 10.0 ml. of nutrient broth. Inoculation of 0.5 ml. of this material (culture D247) intramuscularly into 3 birds approximately 6 weeks of age produced typical synovitis and arthritis. Two of the 3 birds inoculated subcutaneously showed lesions, while the inoculation of 3 birds intratracheally and 3 conjunctivally failed to produce any evidence of the disease. Ten contact control birds failed to show signs after 30 days. The affected birds were destroyed and necropsied (table 1).

On May 19, 1954, another bird was submitted to the laboratory for diagnosis. A sample of the joint exudate was collected aseptically and diluted 1:5 with nutrient broth. This material (culture D278) served as an inoculum for passage in the yolk sac of seven-day embryonating chicken eggs. No antibiotics were added to the material. Embryo mortality occurred on the sixth and seventh day following inoculation and amnioallantoic fluid was harvested. A total of four serial yolk sac passages was made. None of the harvests from these passages showed any hemagglutinating

TABLE 1—Pathogenicity of an Arthritis-Producing Agent in Chickens

No. of culture	Inoculation route	Source of inoculum	No. positive/ No. injected*	Age when injected (weeks)
D–247	Intratracheal	Liver, spleen	0/3	6
D–247	Intramuscular	Liver, spleen	3/3	6
D–247	Subcutaneous	Liver, spleen	2/3	6
D–247	Conjunctival	Liver, spleen	0/3	6
D–247	Contact control	Liver, spleen	0/10	6
D–278	Intramuscular	Liver, spleen	4/4	8
D–278	Intravenous	Liver, spleen	4/4	8
D–278	Subcutaneous	Liver, spleen	2/4	8
D–278	Conjunctival	Liver, spleen	0/4	8
D–278	Intratracheal	Liver, spleen	0/4	8
D–278	Contact control	None	0/4	8

* Birds held for 21 days.

activity nor did they show any evidence of bacterial growth when cultured on blood agar of PPLO agar. A pool of liver and spleen tissue suspension was injected into apparently healthy chickens 8 weeks of age. All 4 of the birds which received 0.5 ml. intramuscularly, or 0.5 ml. intravenously, showed signs of the disease in seven to 14 days. Two of 4 birds which received subcutaneous injections became affected while those inoculated conjunctivally or intratracheally, and the 4 contact controls, failed to respond (table 1).

On Aug. 3, 1954, eight 12-week-old chickens were sent to the laboratory for diagnosis. All birds showed the typical signs and pathological changes of arthritis. Samples of joint fluid were collected and pooled. Bacteriological cultures of this material (culture 33) on blood agar and PPLO agar were negative. After diluting the joint exudate 1:5 with physiological saline, it was injected into the yolk sac of eight, 7-day-old embryonating chicken eggs, each receiving 0.2 ml. of inoculum. Edema of the chorioallantoic membrane with the presence of numerous typical grey-white plaques, especially along the blood vessels, were present. Other pathological changes were enlargement of the liver and spleen. The liver was frequently spotted with small, necrotic, yellow-grey foci and this organ had a definite, green discoloration. The kidneys of some embryos were congested and swollen. The first death of embryos occurred on the fourth day following inoculation, and all embryos had died by the seventh day. This strain (33) of the arthritis agent has remained alive after 72 days in a commercial-type freezer at −15 C.

Bacteriological cultures and hemagglutination tests were conducted on every sample of harvested amnioallantoic fluid. The following carbohydrate mediums were inoculated directly from yolks harvested from the first as well as from the third embryonating egg passage: galactose, mannitol, sucrose, sorbitol, raffinose, dextrose, lactose, rhamnose, maltose, and adonitol. No change in pH was noticed and no turbidity appeared.

Five similar isolations of an agent have been made in embryonating chicken eggs from the joint exudate of field cases (numbers 33, 91, 96, 97, and 105). All of these strains produced mortality when injected into the yolk sac of 7-day embryonating chicken eggs, and all strains reproduced the disease in chickens when injected intravenously or intramuscularly. The typical mortality pattern in embryonating eggs is shown (table 2) with a summary of the total mortality produced by five of the strains (table 3). It is of interest that strain 33 was the most pathogenic with 100 per cent mortality, while strain 97 was the least pathogenic with 73 per cent mortality.

Filtration experiments using Seitz filters have been accomplished with five strains. In these trials, Seitz pads and filters of the vacuum type were used. The filtrate from pads of 5.0 μ and 2.0 μ produced mortality when injected into embryonating chicken eggs, while the filtrate from

TABLE 2—Mortality with Strain 91 of Infective Agent When Inoculated into the Yolk Chamber of 7-Day Embryonating Eggs

Passage No.	Date	No. of eggs inoculated	Days postinoculation											Total
			1	2	3	4	5	6	7	8	9	10	11	
1	11/5	4	0	1	0	0	0	0	1	0	1	0	1	4/4*
2	11/16	4	1	0	1	0	0	0	1	1	....	....	....	4/4
3	11/19	10	0	1	0	0	3	4	1	1	....	....	....	10/10
4	11/30	4	0	1	0	0	0	0	0	1	1	1	....	4/4
5	12/21	4	1	0	0	0	0	1	0	0	0	1	0	3/4
6	1/11	4	0	1	1	0	0	1	0	0	1	....	....	4/4
7	1/25	4	0	0	0	0	0	3	1	....	....	....	....	4/4
8	2/15	12	0	0	0	0	1	2	0	2	1	1	0	7/12
9	3/4	8	0	0	0	0	1	1	1	2	2	0	1	8/8
10	3/18	6	1	0	0	0	0	1	0	0	3	1	....	6/6
11	3/29	5	0	0	1	0	1	0	2	0	1	....	....	5/5

* Number dead over number inoculated.

the pore size of 0.1 μ did not produce mortality. All five strains gave similar results (table 4).

The lethal dose (l.d.)$_{50}$ of these five strains in embryonating eggs was variable. At intervals during the serial yolk sac passages, titrations were made using serial log dilutions of the inoculum. These titrations showed the l.d.$_{50}$ for strain 33 to be 10^{-4} at the level of the third embryo passage and $10^{-2.9}$ at the fifth embryo passage. These data and those of the titrations of the other strains is summarized (table 5).

TABLE 3—Results of Embryo Passages of Five Strains of Infective Agent

No. of culture	No. of serial yolk passages	Eggs inoculated (No.)	Mortality (No.)	Mortality (%)
33	10	101	101	100
91	11	58	52	89
96	10	73	58	79
97	7	37	27	73
105	7	40	30	75

Four of these isolates were tested for their resistance to heat. Yolk material representing strains 33, 91, 96, and 105 was placed in test tubes and incubated at 37 C. for 0, 1, 2, 3, 4, and 5 hours. This material produced mortality after injection into embryonating chicken eggs. Strains 91 and 105 remained viable after exposure to 37 C. for six hours. Incubation of samples at 56 C. and testing at hourly intervals showed that the agent was almost completely destroyed during the first hour (table 6).

Tests were conducted to ascertain the portion of the embryonating egg which contained the highest concentration of the agent. From two embryonating eggs, inoculated with strain 33, harvests of the chorioallantoic fluid, chorioallantoic membrane, embryo, and yolk were made. These materials were prepared as separate inoculums and titrated in embryonating chicken eggs. The yolk material produced the highest l.d.$_{50}$, namely 10^{-4}. The other titers produced were: chorioallantoic fluid, $10^{-7.67}$; chorioallantoic membrane, $10^{-1.00}$; and embryo tissue, $10^{-1.33}$.

The agent was susceptible to certain antibiotics when tested *in vitro*. In these trials, the antibiotics were added to the inoculum and incubated for 30 minutes at room temperature before injection into the yolk chamber of embryonating chicken eggs. Of five strains tested (33, 91, 96, 97, and 105), strain 33 has shown more resistance than the others. Penicillin in amounts of 20,000, 40,000, and 50,000 units per milliliter of inoculum had little, if any, effect on the mortality pattern of embryonating eggs. Streptomycin, terramycin,® achromycin,® and furazolidone® in various amounts did reduce embryo mortality (table 7). Strain 33 continued to produce embryo mortality in the presence of all levels of streptomycin, terramycin, and furazolidone used. Achromycin reduced the embryo mortality by this strain more than any agent tested. Although there is no controlled experimental work regarding the efficiency of these drugs in treating field outbreaks, the use of 200 Gm. of aureomycin or 200 Gm. of furazolidone per ton of feed has been used. Reports indicate that these treatments have given beneficial results (table 7).

TABLE 4—Filtration Experiments with Five Strains of Infective Agent

Filter pore size	Cultures 33	91	96	96	105
Unfiltered	4/4*	5/5	4/4	6/6	5/5
5.0 μ	4/4	3/4	.....	.....	3/5†
2.0 μ	5/5	2/4	4/5	5/6	5/5
0.1 μ	0/4	0/4	1/4**	.....	0/5

* Number deaths/number embryonating eggs inoculated; ** no visible lesions; † other 2 showed lesions.

TABLE 5—Titration of Five Strains of the Infective Agent

Agent (No.)	e.l.d.*$_{50}$ 1st titration trial	e.l.d.$_{50}$ 2nd titration trial
33	10^{-4} (3rd)**	$10^{-2.9}$ (5th)
91	10^{-2} (8th)	.....
96	$10^{-3.16}$ (5th)	$10^{-1.50}$ (9th)
97	$10^{-3.0}$ (6th)	$10^{-2.0}$ (6th)
105	$10^{-1.33}$ (5th)	.....

All titrations made from yolk sac harvest of embryo-adapted material as indicated above.

* Embryo lethal dose; ** numbers in parentheses indicate the number of serial passages in embryonating chicken eggs prior to titration.

TABLE 6—Resistance to Heat of Four Strains of Infective Agent

Treatment of agent Time (hr.)	Temp. (C.)	Cultures treated 33	91	96	105
None		5/5*	5/5	4/5	5/5
1	37	5/5	5/5	5/5	5/5
2	37	5/5	5/5	5/5	5/5
3	37	5/5	5/5	5/5	4/5
4	37	5/5	5/5	5/5	5/5
5	37	5/5	5/5	4/5	5/5
6	37	....	5/5	....	4/5
1	56	0/4	3/5	1/5	1/5
2	56	0/4	1/5	1/5	1/5
3	56	0/4	....	0/5	....
4	56	0/4	....	0/5	....
5	56	0/4	....	0/5	....

* Number deaths/number embryonating eggs inoculated.

DISCUSSION

It appears that the agent involved in this form of arthritis and synovitis is different from the bacterial type reported previously by various investigators. Although only preliminary filtration tests are reported, the size of the agent apparently places it in the classification of a small bacterium or large particle virus.

There is no experimental evidence to in-

TABLE 7—Susceptibility of Strains of Infective Agent to Certain Substances

Drug	Dose/ml. of inoculum	Strain of agent				
		33	91	96	97	105
Penicillin	20,000 units	5/5*	3/5	5/5	3/5	5/5
Penicillin	40,000 units	5/5	2/5	4/4	3/5	3/5
Penicillin	50,000 units	3/3	3/5	3/5	4/5	2/4
Streptomycin	10.0 mg.	4/4	2/4	2/4	0/5	2/4
Streptomycin	20.0 mg.	4/5	0/5	0/4	0/5	4/5
Streptomycin	50.0 mg.	5/5	0/5	0/5	0/5	0/5
Terramycin	10.0 mg.	5/5	0/5	0/5	0/5	0/5
Terramycin	15.0 mg.	5/5	0/5	0/4	1/4	0/5
Terramycin	20.0 mg.	4/4	....	....	....	....
Terramycin	25.0 mg.	....	1/5	0/4	2/4	0/4
Terramycin	50.0 mg.	4/4	2/5	0/4	1/4	1/5
Achromycin	10.0 mg.	3/4	0/5	....	0/5	0/5
Achromycin	15.0 mg.	2/5	1/5	0/3	0/4	....
Achromycin	20.0 mg.	0/5	....	....	....	....
Achromycin	25.0 mg.	....	0/5	0/4	0/4	0/5
Achromycin	50.0 mg.**	2/4	2/5	1/5	2/3	0/5
Furazolidone	0.4 mg.	....	5/5	1/4	0/5	0/5
Furazolidone	0.5 mg.	4/6	....	....	....	....
Furazolidone	0.6 mg.	....	1/4	0/4	0/4	0/4
Furazolidone	1.0 mg.	5/5	....	....	....	....

* Number deaths/number inoculated 7-day embryonating eggs; ** approximate toxic level.

dicate that there is an etiological connection between this disease and chronic respiratory disease or the hemorrhagic syndrome. Intratracheal and conjunctival routes of inoculation have failed to produce any pathological change in chickens. No growth was evident when cultures were made on PPLO agar.

It is also evident from preliminary trials that the agent is not susceptible to penicillin, but some strains are susceptible to achromycin, terramycin, streptomycin, and furazolidone. Reports from the field suggest that aureomycin at the rate of 200 Gm. per ton and furazolidone, 200 Gm. per ton, have been successful in controlling some outbreaks of this disease. However, additional studies will be necessary to determine the degree of therapeutic value for these drugs.

Passage from bird to bird, or bird to embryo has been successful. There is some evidence in our work that embryo passage may reduce the agent's pathogenicity for birds.

SUMMARY

The isolation of an agent or agents from field cases of an arthritic condition in chickens is reported. The agent appears to be similar to that reported by Wills [6] and Olson *et al.*[5]

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An Intracerebral Inoculation Test for Determining the Safety of Newcastle Disease Vaccines

R. P. HANSON, Ph.D.

Madison, Wisconsin

STRAINS of Newcastle disease virus used as living vaccines can be divided into two types; those that can be applied with reasonable safety to chicks as young as 1 day of age and those of somewhat greater pathogenicity that can be given with safety only to chickens 4 to 5 weeks of age or older. Strains of the first type, lentogenic in nature, take longer to kill embryonating eggs, are administered primarily by the respiratory route, and presently are represented by B1, LaSota, and F strains.* Those of the second type, mesogenic in character, kill after a shorter incubation period than the lentogenic type and are introduced into the skin or muscle tissues. These include Roakin and MK 107 (L) strains.*

Contamination of a vaccine representing a lentogenic strain of virus with a mesogenic virus has led to a high mortality among baby chicks (unpublished). A sure and sensitive method of distinguishing between the two types is obviously desirable.

Lentogenic strains not only take longer to kill embryos but also appear unable to multiply in the central nervous system of the chicken. Virus can be recovered from chicks inoculated when less than a week old if they are killed three to seven days later, but the virus has not been recovered by subsequent blind passages of the brain material in chicks. Mesogenic strains, while usually unable to traverse the blood-brain barrier readily multiply in the brain of chickens of any age if introduced by the intracerebral route. Such strains may be propagated in serial brain passages and are usually fatal to mature as well as to day-old chickens. Intracerebral inoculation of virus into day-old chickens is a sensitive method of distinguishing these two types of Newcastle disease virus.

Embryo-cultured virus samples may be used for testing or a vaccine sample may be reconstituted so that 100 doses of vaccine are contained in 1 ml. of diluent. The inoculum is a 1:10 dilution of the sample and since each chick receives 0.05 ml. of the inoculum, intracerebrally, it receives the equivalent of 0.5 dose of vaccine. This quantity may represent from 50 to 500,000 embryo lethal dose $(L.d.)_{50}$ doses. Ten chicks are inoculated with the sample and placed in a Horsefall-Bauer unit; the observation period is eight days. The intracerebral pathogenicity index is calculated on the basis of a daily record of signs and mortality.

Death of the chick (table 1) was given twice the weight that was given to signs of disease. This is similar to the method of weighing severity of disease used for influenza infections of mice. The eight-day observation was selected after trying longer and shorter periods. With rare exception, signs reached a maximum before eight days, frequently at seven days but not often before six. An additional day or two of observation added little information. It should be pointed out that if the index was calculated using the data in

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* Full designations of the strains are: B1, also known as the Blacksburg or intranasal strain, New Jersey-LaSota-1946, England-F, New Jersey-Roakin-1946, Mass-MK 107-1946 (L).

TABLE 1—Data on and Calculations of the Intracerebral Pathogenicity Index Using Inoculum Sample 505

	Days after inoculation											
	1	2	3	4	5	6	7	8	Sum	Factor	Total	Calculation
Dead	0	0	0	1	9	10	10	10	40	2	80	90
Signs	0	0	0	9	1	0	0	0	10	1	10	— = 1.1
Normal	10	10	10	0	0	0	0	0	30	0	0	80
Observations	10	10	10	10	10	10	10	10	80		90	

table 1, but over a shorter or longer period, it would be altered slightly. Therefore, such indexes should not be considered comparable—the index for seven days would be 1.0 and for nine days would be 1.2. The same thing would be true if other factors in the test, the number of chickens and size of dose, were altered.

TABLE 2—Representative Intracerebral Pathogenicity Indexes Obtained with Samples from Repository Stocks and Commercial Vaccines.

Lentogenic strains				Mesogenic strains		
B1		F	LaSota	Roakin		MK 107(L)
0.06	0.00	0.00	0.11	0.60	1.17	1.7
0.00	0.10	0.00	0.00	1.66	1.10	1.5
0.16	0.06	0.15	0.00	1.62	0.83	1.4

Tests on samples of vaccine strains obtained from different sources indicate that the differences between types are of a significant magnitude. The intracerebral pathogenicity index (IPI) of a lentogenic strain should be within the range of 0.0 to 0.25 and that of a mesogenic strain between 0.50 and 1.80.

One must also know the relationship between concentration of the virus and its effect on the chick if the index is to be used to differentiate between lentogenic and mesogenic strains.

The quantity of virus introduced does not markedly affect the index (table 3) if it contains more than 10 l.d.$_{50}$. Hundredfold dilutions of the virus containing from 1 to 1,000,000 embryo l.d.$_{50}$ were inoculated into day-old chicks and the intracerebral pathogenicity indexes derived.

TABLE 3—Effect of Dilution of the Inoculum on Its Intracerebral Pathogenicity Index

	Intracerebral pathogenicity index	
Log embryo lethal dose$_{50}$	Lentogenic strain B1	Mesogenic strain Roakin
0	0.0	0.4
2	0.0	1.0
4	0.0	1.1
6	0.1	1.3

The IPI can be used to distinguish lentogenic and mesogenic strains of Newcastle disease virus over a wide range of virus concentrations. Equally important is the detection of a small quantity of contaminating mesogenic virus in a lentogenic stock. To determine the efficacy of such a detection method, dilutions of Roakin virus containing 10^3, 10^5, and 10^7 embryo l.d.$_{50}$ were mixed with equal quantities of B1 virus containing 10^5, 10^6, and 10^7 and embryo l.d.$_{50}$. The presence of mesogenic virus was readily detectable even when the ratio of mesogenic to lentogenic virus was 1:10,000 although, in this instance, the death time (table 4) was delayed slightly (IPI of 0.51 as compared to 0.76).

TABLE 4—Intracerebral Pathogenicity of Small Quantities of Roakin Strain when Inoculated with Large Quantities of B1 Strain

Log embryo lethal dose$_{50}$ Roakin	Log embryo lethal dose$_{50}$ B1			
	0	5	6	7
0	.....	.....	.....	.....
3	0.76	0.77	0.74	0.51
5	0.92	0.92	1.0	0.81
7	1.1	1.1	1.1	1.0

SUMMARY

The slowly killing or lentogenic strains of Newcastle disease virus appear to be incapable of multiplication in the central nervous system of young chickens. These strains are frequently used as living vaccines for day-old chickens and their contamination with the more pathogenic strains, sometimes used for vaccination of older chickens, can be detected by a test in which day-old chickens are inoculated intracerebrally. The application of the test and its interpretation are discussed.

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Oxytetracycline in Bovine Mastitis. II. Milk Levels Following Local and Parenteral Administration

L. E. BARNES, D.V.M., M.S.

Ames, Iowa

WITH THE introduction of oxytetracycline into the field of veterinary medicine, interest was immediately aroused in its use in the treatment of infectious mastitis. Field trials indicated that it had therapeutic value, but little was known about the concentrations of oxytetracycline in the bovine mammary gland or how long those levels were maintained following administration, either by local infusion or by parenteral injection. This is a report of such a study.

LITERATURE CITED

Schipper and Petersen[12] made a study of the routes and rates of elimination of oxytetracycline when administered intravenously, intramammarily, and intramuscularly in dairy cows. They reported that therapeutic levels were detected in the milk as long as 72 hours following local administration with oxytetracycline ointment. They also found great variations in concentrations between different cows and between quarter samples of the same cow.

Several investigators working independently to establish milk levels of various antibiotics reported an inverse correlation between the volume milk production of the cow and the concentrations of antibiotic found. Kanegis *et al.*,[7] McCulloch *et al.*,[8] Edwards and Haskins,[2] and Schipper and Petersen[12] all reported finding this relationship in milk levels of chlortetracycline. Edwards and Haskins,[2] Jackson and Bryan,[5] Hoerlein and Schalm,[4] Schalm,[10] Jasper and Weirether,[6] Packer,[9] and Throp, Uhrik, and Straley[13] found the same correlation with penicillin milk levels. Trussell and Stevenson[14] and Edwards and Haskins[3] showed the same with streptomycin milk levels.

METHODS OF PROCEDURE

All cows used for determination of milk levels of oxytetracycline were in the dairy herd of the Iowa State College. Animals were chosen as representatives of the following production groups: 10 to 20 lb. per day milk production, 20 to 40 lb. per day milk production, and 40 to 60 lb. per day milk production. All quarters were clinically normal.

Milk samples for assay were collected immediately prior to the regular twice-daily milking. Collection was performed so as to minimize the chance of contamination of the sample. The udder and teats were cleaned in the same manner as has been previously described.[1] Only strict foremilk samples were collected. The samples were identified by number and immediately returned to the laboratory where they were either refrigerated at 7° C. until they could be assayed (usually within six hours) or were frozen for use at a later time.

Oxytetracycline ointment infusions were administered after removal of the milking machine and before the cow left the milking room. A treatment consisted of the administration of the entire contents of one or more commercial ointment infusion tubes (7.1 Gm.), each containing 60 mg. of oxytetracycline per gram of vehicle. The content of one tube was considered as a 400-mg. dose and the contents of two tubes were considered as an 800-mg. dose. Following administration, the teat and udder were briefly massaged with an upward motion to encourage dispersion of the ointment through the quarter.

Quarters which received only one 400-mg. treatment were sampled at milking time every 12 hours for 72 hours following treatment. The quarters receiving three 400-mg. doses at 24-hour intervals were sampled every 12 hours for either 60 or 72 hours following the last treatment. The quarters which were treated with three 800-mg. doses were sampled every 12 hours for 72 hours following the last infusion.

A standard solution of 100 μg. per milliliter of oxytetracycline was prepared by dissolving the contents of one vial of 20,000 μg. of assay quality oxytetracycline in 200 ml. of sterile skim milk. After thorough mixing, this was dispensed into sterile glass vials in 2-ml. amounts and immediately frozen. Serial dilutions of oxytetracycline were used to establish a standard curve and to serve as a control for each assay plate. These dilutions were obtained by serial dilution of the standard solution (100 μg. per milliliter) to concentrations of 80, 40, and 20 μg. per milliliter (fig. 1).

The method of assay employed followed the technique of Vincent and Vincent[16] as modified by Grady and Williams[3] to use large pyrex baking dishes for culture plates. This is ordinarily called the pad-plate method and involves the principle that a given concentration of an antibiotic will always produce the same size zone of inhibition in agar against a test organism under standard conditions. It was found in the present work

From the Department of Veterinary Hygiene, Iowa State College, Ames.

Taken from a thesis submitted to the graduate faculty in partial fulfillment of the requirements for the Master of Science degree, December, 1954.

that oxytetracycline could be measured in whole milk by this method in amounts as small as 1.25 μg. per milliliter.

Two layers of culture medium (table 1) were used in the assay culture plates so as to assure

TABLE 1—Medium for Assay Plate Culture

Peptone	1.2 Gm.
Trypticase BBL*	0.8 Gm.
Yeast extract	0.6 Gm.
Beef extract	0.3 Gm.
Agar	3.0 Gm.
Distilled water q.s. pH 6.5 before autoclaving	200.0 ml.

* Baltimore Biological Laboratory.

uniform thickness of the top layer of agar. Two hundred milliliters of melted medium was poured into a sterile plate with the plate in a level position. After this layer had hardened, 100 ml. of melted medium was inoculated with 0.1 ml. (approximately 2.2×10^6 spores per milliliter) of the suspension of washed spores of *Bacillus cereus* var. *mycoides* (A.T.C.C. 9634, P.C.I. 213) and layered over the top. All plates were poured so that no more than 30 minutes elapsed before the filter-paper pads were placed on the agar. The plates were covered with aluminum wrapping foil to prevent both air-borne contamination and dehydration of the agar during the period of incubation. It was found that prevention of dehydration was the most important consideration.

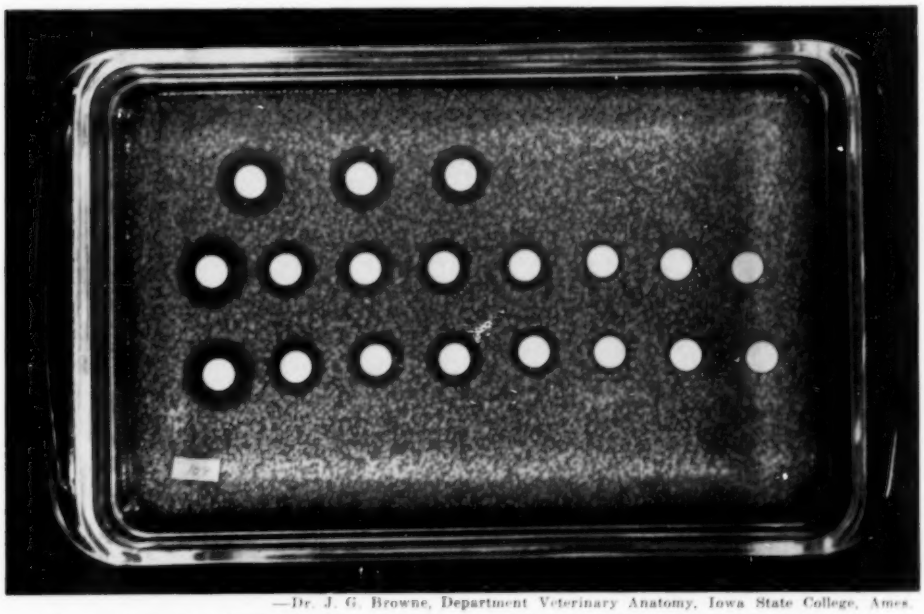
All unknown specimens were diluted with sterile skim milk to reach the range of most accurate readings of the zones of inhibition. This range was found to be between 10 and 30 μg. of oxytetracycline per milliliter. If it was found that the dilution was not in the correct range for accurate interpretation, the sample was reassayed with the dilutions adjusted to bring the readings closer to that range. All samples were assayed in duplicate on each of two plates, using a separate dilution procedure for each plate. A separate series of standard dilutions of oxytetracycline in sterile skim milk was prepared for each assay plate.

The filter pads* were impregnated by immersing them in the sample for 30 seconds and then shaken to remove the excess fluid before placing them on the agar. The plates were incubated at 30 C. for periods varying from 16 to 22 hours. No significant variation in the results were found but the zones were sharper and easier to read if incubation was terminated at 16 hours.

The zones of inhibition were measured by projecting the image of the plate and the zones on a

* No. 740-E, Carl Schleicher and Schuell Co., Keene, N. H.

** Charles Bessler Co., Newark, N. J.



—Dr. J. G. Browne, Department Veterinary Anatomy, Iowa State College, Ames.

Fig. 1—View of an incubated assay plate of the pad-plate method using a pyrex baking dish as the culture plate. The top row of pads were impregnated with sterile skim milk containing 80, 40, and 20 μg. per milliliter (left to right) of oxytetracycline. The pads in the lower rows were impregnated with samples of unknown material in serial dilutions.

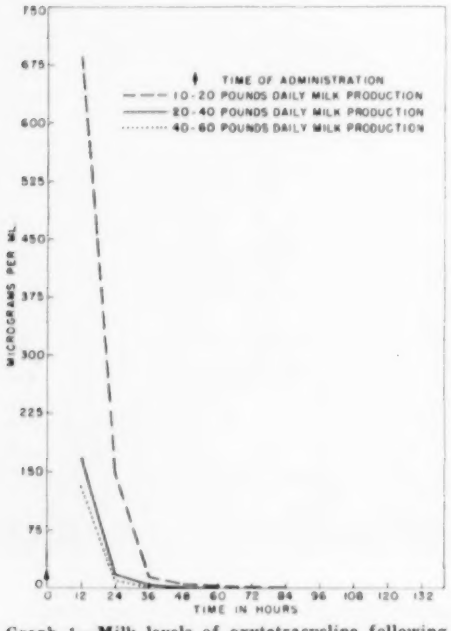
screen with a master vu-graph projector.** The upper mirror of the projector was placed 9 feet 6 inches from the screen. The zone diameters were measured by holding a centimeter scale against the screen in the image of the inhibition zone. It was estimated that the image was magnified 75 times by such projection.

Data for the standard curve were collected by averaging the diameters of the zones of inhibition produced on all plates by the various standard concentrations. These averages were plotted on the logarithmic scale of the abscissa against the zone diameters in centimeters on the ordinate of semilogarithmic graph paper and gave a straight line curve. The measurement of the diameter of the zone formed by unknown material was applied to the scale to find the micrograms per milliliter of oxytetracycline in the diluted sample. This number was multiplied by the dilution factor to give the final reading of micrograms per milliliter of oxytetracycline in the original sample. All of the original samples which contained less than 30 μg. per milliliter of the drug were assayed in duplicate without dilution.

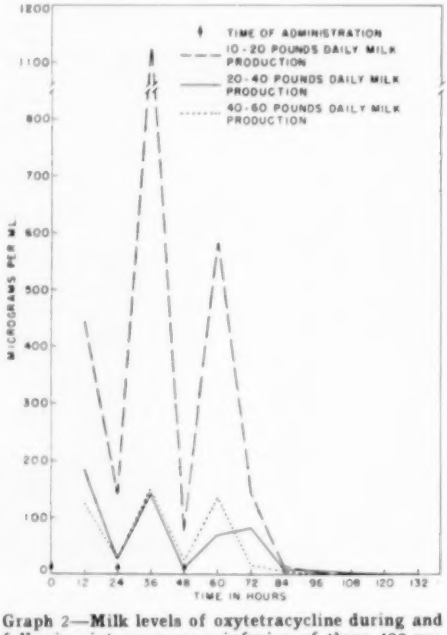
Samples of milk and blood collected from cows which were injected intravenously and intramuscularly with oxytetracycline were assayed by the same method as described above. The blood samples were allowed to clot and the serum was withdrawn for assay. All serum samples were assayed in duplicate on each of two plates using undiluted material.

Results of Assay

All quarters had a detectable level of oxytetracycline for 24 hours or longer following one 400-mg. infusion (graph 1). Oxytetracycline was detected in the milk of nine treated quarters of periods of time ranging from 24 to 72 hours, the average being 37.3 hours. The first samples collected following treatment showed the highest levels and each succeeding sample showed a sharp decrease in concentration. There was much variation in the concentrations found in different cows and in different quarters of the same cow. However, there was not much variation in the length of time that detectable levels were present in different quarters of the same cow or in different cows within the same milk production group. The quarter of the cow with the lowest daily milk production showed the highest levels of oxytetracycline at the various samplings and held a detectable level of the drug for the longest time. The quarters of cows which had the


TIME OF ADMINISTRATION
10-20 POUNDS DAILY MILK PRODUCTION
20-40 POUNDS DAILY MILK PRODUCTION
40-60 POUNDS DAILY MILK PRODUCTION
MICROGRAMS PER ML.
TIME IN HOURS

Graph 1—Milk levels of oxytetracycline following intramammary infusion of one 400-mg. dose.


TIME OF ADMINISTRATION
10-20 POUNDS DAILY MILK PRODUCTION
20-40 POUNDS DAILY MILK PRODUCTION
40-60 POUNDS DAILY MILK PRODUCTION
MICROGRAMS PER ML.
TIME IN HOURS

Graph 2—Milk levels of oxytetracycline during and following intramammary infusion of three 400-mg. doses.

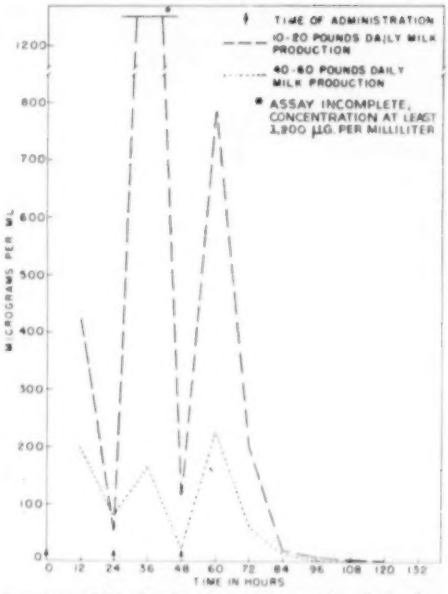
highest daily production showed the lowest levels of drug at the various samplings and retained a level of the drug for the shortest time.

The highest concentrations of oxytetracycline were found 12 hours following each infusion (graph 2). The levels of oxytetracycline found in the samples collected 12 hours following the first dose are comparable with those collected at the same time following the single 400-mg. dose. Following the high level attained after each treatment, the level of oxytetracycline dropped precipitously. Here too, the highest levels were found in samples from the cow having the lowest daily milk production and the lowest levels of drug were found in samples from a cow in the group of highest daily milk production. In general, the detectable levels were found for the longest times in the cows with a low daily milk production and the shortest duration occurred in cows with a high daily milk production.

There did not appear to be a consistent cumulative effect in the milk levels corresponding to repeated doses. Individual levels found following the later doses were not necessarily greater than the levels found following the preceding administration (graphs 2 and 3). Sometimes the increase with each infusion was very evident, especially in quarters of low-producing animals, but in several instances the concentration was nearly the same or even lower in the sample collected 12 hours following the second or the third treatment, as compared to the 12-hour sample following the first dose.

All treated quarters showed a detectable level of oxytetracycline for 24 hours and most of them for at least 36 hours following the last infusion. The longest time that oxytetracycline could be detected in the milk of a treated quarter was 84 hours after the last treatment or a total of 132 hours from the beginning of therapy. The shortest time that it could be found in the milk was 24 hours after the last treatment. The average total time that the drug could be detected in the milk of all quarters was 93.3 hours and the average time detectable levels were found following the last infusion was 45.3 hours.

The milk levels of oxytetracycline which were found in two quarters following three



Graph 3—Milk levels of oxytetracycline following intramammary infusion of three 800-mg. doses.

successive 800-mg. infusions at 24-hour intervals are shown (graph 3). The levels found were considerably greater than those found in the quarters treated with repeated 400-mg. doses. There was no obvious tendency for a cumulative effect with the second and third doses. Following all three treatments there was a marked drop in the concentrations between the twelfth and twenty-fourth hour following the infusion. However, the level at the twenty-fourth hour following each 800-mg. infusion was consistently higher than that found at the same hour following each succeeding 400-mg. dose. Detectable levels of oxytetracycline were found for an average of 54 hours following the last administration and for an average total of 102 hours following the initiation of therapy. There is some correlation between the volume of milk production, the height of drug concentration attained with the dosage, and the length of time it was maintained.

In table 2 are the results of the assay of blood and milk samples from 2 cows following the intramuscular and intravenous administration of 1 Gm. of oxytetracycline. Blood levels were measurable in

TABLE 2—Blood and Milk Levels Following Intravenous and Intramuscular Administration of 1 Gm. of Oxytetracycline

Route	Cow	Daily milk production (lb.)	Material assayed	Time post-treatment (hours) (μg./ml.) 1	2	4	8	12	24	36	48
Intramuscular	3681	30.3	blood	—	1.25	1.25	—	—	—	—	—
			milk	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Intravenous	3554	45.0	blood	8.65	—	trace	—	—	—	—	—
			milk	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

both cows but neither animal showed any detectable amount of oxytetracycline in the milk.

Discussion of Results

As might be expected, the highest concentrations of oxytetracycline were found in the first milk samples collected following administration. From this point, the levels progressively diminished at a rapid rate until the drug could no longer be detected. Following one 400-mg. infusion, all quarters examined demonstrated oxytetracycline for at least 24 hours, with the average time being 37.3 hours. Repeated 400-mg. doses resulted in generally higher levels of the drug than the single dose, but the assay did not show that there was a consistent cumulative effect. Detectable levels were found for an average of 45.3 hours following the last dose and were seen in some instances as long as 96 hours after the last dose. Repeated doses of 800 mg. of oxytetracycline resulted in milk levels which were higher than those following repeated 400-mg. doses, but there was no consistent cumulative effect. Levels were found for an average of 54 hours following the last dose of this schedule. These results, in general, are similar to those obtained by Schipper and Petersen,[12] even though the present assay method does not appear to be as sensitive as theirs.

The results of this investigation show that intramammary infusions of oxytetracycline readily yield concentrations which are inhibitory to the common mastitis organisms with the possible exception of *Pseudomonas aeruginosa*. In many instances, as long as oxytetracycline could be found, it was present in amounts which were inhibitory to the common mastitis organisms. The concentrations found immediately following repeated 400-mg. doses were usually high enough to be lethal to the streptococci but perhaps not to the staphylococci.[1] This may explain, in part, the relatively poor results obtained in the treatment of staphylococcic mastitis in that work. The lowest levels seen between treatments were always sufficient to inhibit but seldom sufficient to kill the staphylococci. This is possibly borne out by the clinical improvement of mastitis cases, whether bacteriological recovery occurred or not.

The levels of oxytetracycline attained and the length of time that they could be detected in the milk appeared to be inversely correlated with the volume milk production of that quarter. Invariably it was found that the milk of the lowest producer showed the highest levels and usually for the longest time following infusion. This information could be used in the field as a guide for the treatment of mastitis cases. Animals in high production probably should be treated more frequently and with dosages higher than 400 mg. to assure adequate therapy, while nonlactating animals, or those in late lactation, could be treated less frequently with a 400-mg. dose. This same inverse correlation between milk production and the levels of antibiotic has been shown by other investigators working with various antibiotics.[2,4,11,13,14] It appears that if a constant high level were desired in a quarter, it would be necessary to repeat the dosage more often than every 24 hours. However, it probably could not be expected that increased frequency or amount of dosage would result in greatly prolonged levels of antibiotic. The expression of a cumulative effect appears to be an increase in concentration rather than a marked increase in the length of time that the drug is present.

Rather extreme variations were found in the oxytetracycline levels of different quarters of the same cow given the same treatment. Schipper and Petersen[12] pointed out similar variations following ointment infusions. No study was made to explain this variation, but possibly it would have been less if the assay samples had been col-

lected midway in the milking instead of using foremilk samples. However, there may also have been other factors involved.

Results of the assay of milk samples collected following intravenous and intramuscular injections of 1 Gm. of oxytetracycline showed that, if present, the levels were less than 1.25 μg. per milliliter with the method used. Schipper and Petersen [12] used 2-Gm. doses of oxytetracycline intramuscularly and intravenously and were able to show that detectable milk levels could be found following the intravenous dose but not following the intramuscular administration.

It is significant that this work demonstrated no transfer of detectable amounts of oxytetracycline from treated to untreated quarters in the same cow with the assay method used. No matter what the amount of infusion into the other quarters or the amount of daily milk production, each quarter appeared to be independent in this regard.

Summary and Conclusions

1) Assay of milk samples following single 400-mg. intramammary infusions of oxytetracycline showed that detectable levels were consistently present for 24 hours or longer with an average time of 37.3 hours.

2) Assay of milk samples following repeated 400-mg. intramammary infusions showed detectable levels for at least 24 hours following the last infusion with an average time of 45.3 hours but did not indicate that there was any consistent cumulative effect as expressed in either concentrations attained or length of time present.

3) Oxytetracycline levels found following repeated 800-mg. intramammary infusions generally paralleled those found with repeated 400-mg. doses but were considerably greater in amount. No consistent cumulative effect as expressed in either concentration or length of time was found with this dosage schedule.

4) Marked variations in the oxytetracycline concentrations were found in different quarters of the same cow and in different cows in the same production groups, but there was not much variation in the length of time that detectable levels were found in those same animals.

5) Milk samples collected following 1-Gm. doses of oxytetracycline intramuscularly and intravenously showed no detectable amounts of drug with the method used.

6) No detectable amounts of oxytetracycline could be found with the assay method used in milk from untreated quarters when the other quarters were treated.

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Experimental Transmission of a Catarrhal Enteritis of Chicks Similar to Bluecomb Disease

J. McN. SIEBURTH, Ph.D., and B. S. POMEROY, D.V.M., Ph.D.

St. Paul, Minnesota

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Beaudette[1] in 1929 described a disorder of chickens, termed "x disease," which was characterized by a catarrhal enteritis and cyanosis of the head. Transmission studies with unfiltered and filtered materials failed to demonstrate an infectious agent. This report was not confirmed or elaborated on until 1940. Since then, many independent papers have been published describing the pathology, hematology, and clinical chemistry of similar conditions under a multitude of different terms, the most common of which are pullet disease, bluecomb disease, and avian monocytosis.[8]

Most workers have found that the transmission of the disease is either impossible or very difficult. Waller[10] and Watanabe[12] have reported a virus etiology. In their experimental studies on transmission, embryonating egg preparations of either blood or organs induced depression and monocytosis in some birds but failed to cause death.

Field outbreaks provide only a seasonal and variable source of infected birds for study. Although attempts to transmit a catarrhal enteritis of turkeys, similar to bluecomb disease, had failed in growing birds,[6] bluecomb disease has been readily transmitted in day-old poults in which mortality can be used as an index of infection.[7]

The purpose of this paper is to describe the experimental transmission of a catarrhal enteritis of young chickens in the day-old chick and to compare this disease with that of turkeys transmitted in the day-old poult.

Methods and Materials

Experimental Procedures.—Day-old New Hampshire, dominant-white, broiler cross, or White Leghorn chicks were obtained from U. S. pullorum-clean hatcheries and housed in wire-floored, electrically heated brooders. An antibiotic-free, practical-type ration containing 25 per cent protein was used. Feed and water were supplied *ad libitum*. Infected groups were maintained in separate cages in a different room to avoid infection of the uninoculated control group.

Infected chicks were killed between the third and fifth day to obtain infective intestinal material. Cultures were made of the liver and intestine for members of the genus Salmonella. Intestines with typical catarrhal enteritis and free of Salmonella were stored in rubber-stoppered tubes at —45 C. until time for use. The small intestines and ceca were minced finely with scissors, triturated with sand, and diluted with saline to make a 10 per cent suspension. Four drops were administered orally as the inoculum to day-old chicks.

Source of Infectious Material.—White Leghorn cockerels and pullets approximately 8 weeks of age, displaying depression, emaciation, cyanosis of the head, and diarrhea, were obtained from a range flock of a U.S.R.O.P.* breeder. Necropsies revealed a catarrhal enteritis without involvement of known intestinal pathogens. Since only intestinal preparations have proved consistently infective in the disease of turkeys,[7] a 10 per cent intestinal suspension was administered orally to 3 White Leghorn pullets 6 weeks of age. Diarrhea was the only sign evident by the fifth day postinoculation. The birds were killed and necropsy showed a catarrhal enteritis. The intestines were used as a source of inoculum for transmission in day-old chicks.

Chicks infected at 1 day of age showed no overt signs other than slight depression, weight loss, and watery droppings. The organs exhibited no gross lesions. The entire intestinal tract from the gizzard to the ileocecal valve was light in color and filled with watery material interspersed with bubbles. The ceca were sometimes distended with the light-colored, watery, foamy contents.

Experimental Results

Mortality.—The mortality among chicks inoculated at 1 day of age in 13 serial transfers is shown in graph 1. On an average, mortality started about the third day, reached 50 per cent by the eighth day, with a leveling off between the tenth and fourteenth day. The lowest mortality occurring by the twelfth day in all 13 experiments was 37 per cent, the highest was 95 per cent, and the average was 67 per cent.

From the School of Veterinary Medicine, University of Minnesota, St. Paul.

Dr. Sieburth's present address is: Animal Pathology Section, Virginia Agricultural Experiment Station, Blacksburg.

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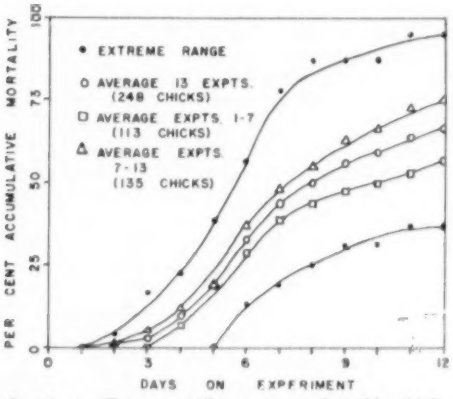
Polyotic was furnished by Lederle Laboratories; vetstrep by Merck and Co.; and bicillin by Wyeth and Co.

* U. S. record of performance.

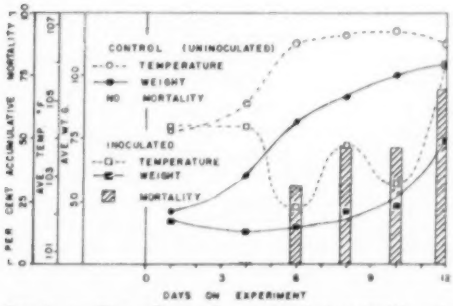
The average mortality for the first seven experiments was slightly lower than for the last six experiments.

The mortality of the disease among the day-old chicks appears to be lower than the disease among the day-old poults. Poults experimentally infected suffered 50 per cent mortality between the fourth and fifth day and 100 per cent by the seventh day postinoculation.


PER CENT ACCUMULATIVE MORTALITY
EXTREME RANGE
AVERAGE 13 EXPTS. (248 CHICKS)
AVERAGE EXPTS. 1-7 (113 CHICKS)
AVERAGE EXPTS. 7-13 (135 CHICKS)
DAYS ON EXPERIMENT

Graph 1—The mortality among day-old chicks inoculated with infective intestinal material of chicken origin.

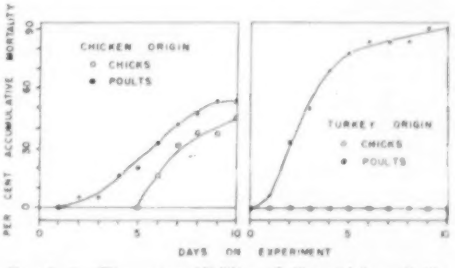
Depression of Temperature and Growth Rate.—The effect of the disease on body weight, temperature, and mortality of day-old chicks is shown in graph 2. The uninfected control birds reached their normal temperature of 106 to 107 F. by the sixth day and exhibited a steady rate of growth. The temperature of the inoculated chicks was markedly depressed, having a low of 102.2 F. on the sixth day, and did not approach normal until the twelfth day. The surviving infected chicks failed to gain weight until after the tenth day when mortality had run its course.



Graph 2—The effect of a catarrhal enteritis of chicks on body weight, temperature, and mortality.

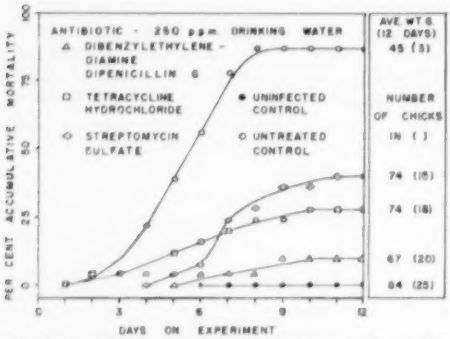
Susceptibility of the Chick and Poult.—Both the chicken and turkey bluecomb inoculums caused similar intestinal lesions, cessation of growth, and temperature depression in the homologous species. These inoculums were also tested in the heterologous species (graph 3). Those from chickens caused an even greater mortality in poults than in chicks and both species had typical intestinal lesions. The turkey-passed, chicken-source material retained its infectivity for chicks. Although the turkey-source material was active against poults, it failed to cause either mortality or typical intestinal lesions in chicks. However, the chick-passed turkey material still retained its infectivity for poults.

Susceptibility to Antibiotics.—Antibiotics have been used widely in the field in attempts to prevent and treat bluecomb disease in both chickens and turkeys. To test the susceptibility of the chick-infective material to various antibiotics, the latter were added to the drinking water at a concentration of 250 p.p.m. of the active material. Four groups of 25-day-old chicks received the same inoculum, one group was left as the untreated control, and one group received each of the following antibiotics: dibenzylethylenediamine dipenicillin G (bicillin oral suspension®), tetracycline hydrochloride (polyotic®), and streptomycin sulfate (vetstrep®). Water was supplied in all-glass founts. The birds were weighed after mortality had ceased on the twelfth day (graph 4). The con-



Graph 3—The susceptibility of the chick and the poult to catarrhal enteritis of both chicken and turkey origin.

trols started dying by the third day, 50 per cent mortality occurred between the fifth and sixth day, and a maximum mortality of 87 per cent by the eighth day after inoculation. Mortality was below 40



Graph 4 — The effect of antibiotic-supplemented drinking water on the mortality and weight of infected chicks.

per cent in the treated group. Dibenzylethylenediamine dipenicillin G appeared to be the most effective agent, the birds receiving it suffering only 10 per cent mortality. The untreated chicks failed to gain weight, while those receiving antibiotic gained weight although not to the same extent as the control chickens.

Miscellaneous Observations.—There appeared to be no appreciable difference in the susceptibility of various breeds. Day-old New Hampshire, dominant-white, broiler cross, and White Leghorn chicks suffered similar mortality. Both male and female chicks were susceptible to the infection.

In an attempt to increase the mortality, feed was withheld from certain lots of chicks until 18 hours postinoculation. Mortality among these was delayed but eventually reached the same level as among those put on feed at the time of inoculation.

Intestines from chicks having typical lesions were stored at —45 C. and were infective for day-old chicks as long as 134 days.

Discussion

Although bluecomb disease of chickens has been reported in birds as young as 4 weeks of age,[4] only pullets have been used for experimental work. Mortality from bluecomb disease in this age group during field outbreaks is usually low—from 5 to 20 per cent. All pullets were not susceptible[2,11] to experimental infection and infection was not lethal.[11,12] The criteria of infection were clinical signs or histopathological and hematological changes.

For continuing investigation, one should have a clinically apparent and reproducible infection with convenient criteria of infection. The day-old chick of either sex, inoculated orally with infective intestinal material provides these criteria. A catarrhal enteritis is apparent within 36 hours, and the chicks become emaciated by the third day, when mortality starts. The infection has been consistently passed through 15 serial transfers with an average mortality of 50 per cent by the eighth day and 67 per cent by the twelfth day.

Peterson and Hymas[6] reported that growing turkeys, naturally infected with bluecomb disease, developed subnormal temperatures as well as loss in weight. Day-old poults infected experimentally with turkey bluecomb disease also showed a marked temperature depression and failed to gain weight.[8] Day-old chicks infected with material of chicken origin similarly exhibited a characteristic depression of temperature and growth rate.

Another similarity of the catarrhal enteritis in chickens and turkeys is response to antibiotic treatment. Individual treatment of naturally infected growing birds with antibiotics has been reported to stop death losses and to speed recovery in both chickens[5] and turkeys.[6] Antibiotic supplementation of the feed[7] and the water[9] also alters the course of the disease in poults naturally infected at 1 day of age. The chick disease described here may also yield to antibiotics. The untreated group suffered a high mortality and survivors failed to gain weight during the experimental period. With antibiotic-supplementation of the drinking water, started immediately after exposure and continued 12 days, the mortality was reduced by 50 to 85 per cent and the infected chicks gained weight.

Bluecomb disease of chickens and that of turkeys have many characteristics in common. One might expect that if the etiological agent was the same the diseases would be infectious for the heterologous species. However, chicks were not infected by contact with morbid poults in a turkey blue-

comb-infected brooder house[7] and highly infective poult intestinal material likewise failed to cause an apparent infection when administered orally to day-old chicks in the laboratory. The converse, however, was not true. Intestinal material from chicks was even more infectious for poults than for chicks. This suggests that the etiology of the two diseases may not be identical. Whether poults, recovered from either chicken- or turkey-source infection, are susceptible to the heterologous infection should be investigated. The true relationship of so-called bluecomb in the two species, however, will not be understood until the characteristics of the etiological agent have been ascertained.

Chick-infective material appeared to be less susceptible to deterioration under refrigeration than poult-infective material. Infective chick intestines stored in rubber-stoppered tubes at —45 C. maintained their infectivity for at least 134 days. Poult intestines infected with the turkey disease lost their infectivity under these conditions after 60 days.

SUMMARY

1) A catarrhal enteritis of chickens resembling bluecomb disease was readily transmitted through 15 serial passages in the day-old chick by the oral administration of suspensions of infected intestines.

2) Infected chicks developed a catarrhal enteritis as well as a characteristic depression of temperature and growth rate.

3) Mortality ranged from 37 to 95 per cent with an average of 67 per cent during an experimental period of 12 days.

4) When the drinking water was supplemented with certain antibiotic agents the mortality was reduced and infected chicks gained weight even though slightly.

5) Unlike turkey bluecomb disease which appeared to be species specific, the disease of chicken origin seemed to affect poults as easily as chicks.

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Tocopherol Levels of the Early Milks of Semirange Cattle

KARL F. SWINGLE, Ph.D.; JOHN W. SAFFORD, D.V.M.;
DONALD E. McROBERTS, B.S.

Bozeman, Montana

WHITE MUSCLE disease (also known as nutritional muscular dystrophy) has been observed almost every spring for 15 years among the calves of certain beef herds in the Sula Basin, a mountain valley in western Montana. Other herds in the same valley, apparently operating under similar conditions, have never experienced losses from this malady.

The cattle graze in the mountains from May until the latter part of October, when they are moved to meadows from which the hay has been harvested, remaining there until the next May. The only feed the cows receive during the winter is the hay that has been grown on the irrigated meadows. It is composed of timothy, brome grass, bluegrass, redtop, and carex, with a little alfalfa. It had been cut, sun cured, and stored in stacks until fed by scattering on the ground or snow. The cows are at no time confined to barns or sheds, and they have their calves in the open meadow in March, April, or early May. The water is supplied from mountain streams which flow through the meadows.

White muscle disease in calves has been associated with a vitamin E deficiency on the basis of four types of evidence: (1) The incidence and severity of the disease have been reduced by feeding *alpha*-tocopherol (vitamin E) to the preparturient cow or the calf or both.[4, 7, 14, 22, 25, 26] (2) The signs and lesions of the disease in calves resemble those produced in laboratory and farm animals by vitamin E-deficient diets.[2] (3) Analysis of the rations of the cows in white muscle areas has shown a lower level of vitamin E than was found in the rations of cows in neighboring areas free of the disease.[4] (4) The feeding, to young calves, of experimental rations known to be deficient only in *alpha*-tocopherol has resulted in the development of typical white muscle disease.[5, 6, 10, 22]

Additional evidence for the role of vitamin E deficiency in producing muscular dystrophy in calves would be derived from a knowledge of the amount of this vitamin supplied to the calves in the colostrum and milk of their dams. The vitamin E level in the colostrum and milk of a number of species of mammals has been determined. This value has been previously reported for human milk,[1, 8, 11, 16, 24, 29] for ewe milk,[28, 40] for goat milk,[24, 28] for sow milk,[28] and for dairy cow milk.[1, 3, 9, 12, 13, 16–23, 25–29, 37, 39] However, the calves of dairy cows have seldom been reported to develop muscular dystrophy, and the only data on the milk tocopherol levels of beef cows are from 3 cows known to have produced dystrophic calves.[21]

For this reason, a study was designed to give additional data on the colostrum and milk tocopherol levels of beef cows from herds where muscular dystrophy is common and from similar herds where the disease is not found. The results of that experiment are reported in this paper.

EXPERIMENTAL PROCEDURE

The cows were selected at random from the various herds. Colostrum samples were collected on the day of parturition and daily for the next three days. Thereafter, milk samples were taken on days 8, 12, 16, 20, 24, 28, and 32 after calving. The samples were poured into 4-ounce screw-cap bottles containing 2 drops of formalin. They were shipped to the laboratory promptly and stored in the refrigerator until analyzed. Milk tocopherol analyses were made according to the methods described by Quaife[29] with the microhydrogenation technique of Quaife and Bichler.[30] In addition, many of the 1954 samples were analyzed for total fat by weighing the residue left after evaporating the solvent from the hexane extract of the milk.

Ten cows in 1953 and 5 cows in 1954 were milked from herd 1, which contained 275 Hereford cows. The losses from white muscle disease in the calves of this herd for the past ten years were from none to 25 per cent.

Ten cows in 1953 and 2 cows in 1954 were milked from herd 2, which contained 150 Hereford cows. The losses from white muscle disease in the calves of this herd in the past 15 years had been from 5 to 30 per cent, with an average of 18 per cent.

Nine cows in 1953 and 10 cows in 1954 were

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The volunteer assistance of David D. Casebolt in collecting the milk samples from the college dairy herd is also gratefully acknowledged.

Our thanks go also to the late Dr. John Macho of Hamilton, Mont., who helped in organizing the study, and who stood by for clinical and necropsy services when they were required.

milked from herd 3 which contained 65 Hereford cows. Two years previously a 3 per cent loss from white muscle disease had been experienced. The year before that the loss was 15 per cent.

Three cows in 1953 and none in 1954 were milked from herd 4 which contained 200 Hereford cows. No losses from white muscle disease in calves have been experienced on this ranch.

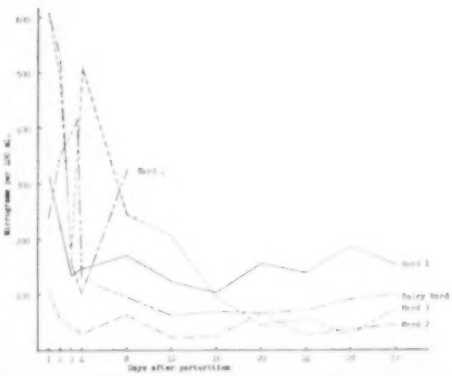
One cow in 1953 and 3 in 1954 were milked in herd 5 which contained 150 cows. Losses from white muscle disease have not occurred on this ranch.

For purposes of comparison, 5 cows from the dairy herd of the Montana State College were milked on the same schedule as the cows from Sula Basin, and the milk samples were analyzed for total tocopherol.

All the milk samples, including those from the dairy cows, were taken by separating the cows from their calves for a short time and then drawing up to 30 ml. of milk from each quarter before returning the cows to their calves or, in the case of the dairy cows, before proceeding with the regular milking.

RESULTS AND DISCUSSION

The results of the tocopherol and fat analyses of the colostrum and milk samples from the five beef herds from the Sula Basin are given (tables 1 to 5). In table 6 are recorded the tocopherol levels found in the colostrum and milk of the 5 dairy cows from the college herd. For the samples in which the fat was determined, the values of the tocopherol in terms of micrograms per gram of fat were calculated, and the results of these calculations are given (table 7).



Graph 1—Average colostrum and milk tocopherol levels of four beef herds and one dairy herd, 1953.

The changes in average tocopherol level of each herd from parturition through 32

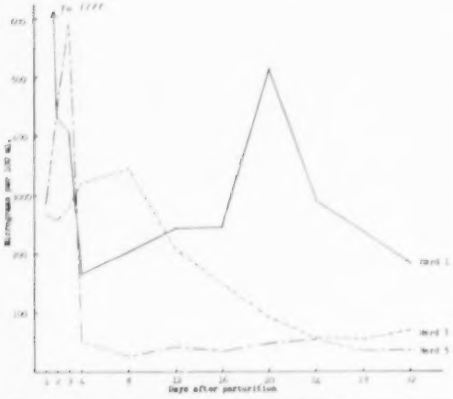
TABLE 1—Tocopherol and Fat Analyses of Milks from Herd 1

Days after calving	Cow number 1		2		3		4		5		6	7	8	9	10	Toc. herd av.
	Toc.*	Fat*	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Toc.	Toc.	Toc.	Toc.	
1953																
1	450	.....	10	.....	155	.....	185	.....	240	.....	915	230	465	140	360	315
2	155	.....	140	.....	10	.....	495	.....	300	.....	680	50	130	55	195	222
3	420	.....	135	.....	10	.....	100	.....	170	.....	75	5	300	30	115	136
4	285	.....	115	.....	145	.....	120	.....	35	.....	55	115	355	75	175	148
8	240	.....	35	.....	190	.....	85	.....	255	.....	117	85	190	360	155	171
12	185	.....	25	.....	.....	.....	80	.....	115	.....	69	210	130	145	150	123
16	130	.....	15	.....	70	.....	30	.....	40	.....	185	90	265	.....	110	103
20	60	.....	235	.....	180	.....	175	.....	175	.....	135	130	310	.....	110	157
24	110	.....	180	.....	160	.....	110	.....	130	.....	280	75	165	.....	55	140
28	145	.....	390	.....	100	.....	85	.....	.....	.....	.....	.....	90	.....	315	188
32	135	.....	145	.....	105	.....	110	.....	165	.....	.....	50	165	.....	375	156
Av. colos.	328	.....	100	.....	80	.....	225	.....	186	.....	431	100	312	75	211	205
Av. milk	144	.....	146	.....	134	.....	96	.....	147	.....	157	107	188	252	181	155
1954																
1	3957	9.80	461	1.90	516	3.20	3680	6.80	272	2.20	.....	.....	.....	.....	.....	1777
2	377	3.60	488	1.30	638	3.50	244	2.50	411	4.90	.....	.....	.....	.....	.....	432
3	849	5.60	849	7.80	147	2.60	119	1.40	69	.....	.....	.....	.....	.....	.....	407
4	244	7.50	147	3.10	105	3.30	161	1.60	180	.....	.....	.....	.....	.....	.....	167
8	254	.....	28	.....	412	.....	120	.....	199	.....	.....	.....	.....	.....	.....	203
12	745	.....	153	.....	245	.....	42	.....	42	.....	.....	.....	.....	.....	.....	245
16	167	.....	291	.....	430	.....	37	.....	305	.....	.....	.....	.....	.....	.....	246
20	97	.....	222	.....	208	.....	250	.....	1804	.....	.....	.....	.....	.....	.....	516
24	541	.....	222	.....	167	.....	291	.....	236	.....	.....	.....	.....	.....	.....	291
28	250	.....	111	.....	402	.....	194	.....	.....	.....	.....	.....	.....	.....	.....	239
32	111	.....	153	.....	125	.....	111	.....	420	.....	.....	.....	.....	.....	.....	184
Av. colos.	1357	.....	486	.....	352	.....	1051	.....	233	.....	.....	.....	.....	.....	.....	696
Av. milk	309	.....	169	.....	284	.....	149	.....	501	.....	.....	.....	.....	.....	.....	275

* Tocopherol expressed as micrograms per 100 ml. of milk and fat as percentages.

days of lactation are plotted (graph 1 and 2). Only herds from which 3 or more cows were sampled are included.

None of the herds from which these samples were taken produced any dystrophic calves in either 1953 or 1954, the years in which these studies were made. As a matter of fact, muscular dystrophy was almost nonexistent in that part of Montana for the first time in many years.



Graph 2—Average colostrum and milk tocopherol levels of three beef herds, 1954.

The colostrum from dairy cows has been reported by Parrish *et al.*[28] to contain from 77 to 3,820 μg. of tocopherol per 100 ml., while Kieferle *et al.*[15] gave a range of 300 to 400. When the colostrum tocopherol value is expressed as micrograms per gram of fat, Blaxter *et al.*[3] gave a value of 73 to 368 and Parrish *et al.*,[27] a value of 57 to 117. The values obtained from the analysis of the colostrum of the college dairy herd ranged for individual samples from 40 to 1,100 μg. per 100 ml., with an average value of 283. Individual samples from the 5 Hereford herds ranged from 0 to 3,957 μg. per 100 ml., or from 22 to 1,413 μg. per gram of fat. Individual cow averages for the four colostrum samples varied from 18 to 1,537 μg. per 100 ml. of colostrum, or from 40 to 737 μg. per gram of fat. The colostrum tocopherol averages for the five herds sampled in 1953 were: 205, 59, 446, 390, and 34 μg. per 100 ml., respectively. In 1954 they were: 696, 289, 282, (herd 4 not sampled), and 319 μg. per 100 ml. The values expressed as micrograms

TABLE 2—Tocopherol and Fat Analyses of Milks from Herd 2

Days after calving	Cow number 1 Toc.*	1 Fat.*	2 Toc.	3 Toc.	4 Toc.	5 Toc.	6 Toc.	7 Toc.	8 Toc.	9 Toc.	10 Toc.	Toc. herd av.
1953												
1	0	.....	130	65	195	185	345	45	30	40	71	111
2	195	.....	100	55	35	0	30	....	90	10	50	59
3	10	.....	35	0	0	145	5	35	25	10	136	40
4	65	.....	65	15	20	35	5	5	25	10	60	30
8	330	.....	55	15	5	45	....	20	32	15	60	64
12	0	.....	30	20	10	75	10	25	45	0	15	23
16	10	.....	20	10	5	75	15	15	20	60	15	24
20	460	.....	60	15	5	25	10	10	25	15	40	67
24	55	.....	60	35	5	5	0	40	30	35	25	29
28	90	.....	40	5	50	30	55	65	15	15	15	38
32	110	.....	25	50	35	60	50	30	25	25	....	46
Av. colos.	60	.....	82	34	62	91	96	28	42	18	79	59
Av. milk	151	.....	41	21	16	45	23	29	27	24	28	40
1954												
1	710	3.77	....	....	....	....	....	....	....	....	....	....
2	63	2.67	....	....	....	....	....	....	....	....	....	....
3	94	2.70	....	....	....	....	....	....	....	....	....	....
4	.....	.....	....	....	....	....	....	....	....	....	....	....
8	.....	.....	28**	....	....	....	....	....	....	....	....	....
12	.....	.....	28**	....	....	....	....	....	....	....	....	....
16	.....	.....	19**	....	....	....	....	....	....	....	....	....
20	.....	.....	14**	....	....	....	....	....	....	....	....	....
24	.....	.....	42**	....	....	....	....	....	....	....	....	....
28	.....	.....	....	....	....	....	....	....	....	....	....	....
32	.....	.....	....	....	....	....	....	....	....	....	....	....
Av. colos.	289	.....	....	....	....	....	....	....	....	....	....	....
Av. milk	.....	.....	26	....	....	....	....	....	....	....	....	....

* Tocopherol expressed as micrograms per 100 ml. of milk and fat as percentages. ** These samples were received at one time. The dates of their collection are unknown. This cow's calf was suspected of having white muscle disease.

TABLE 3—Tocopherol and Fat Analyses of Milks from Herd 3

Days after calving	Cow number																				Toc. herd av.
	1		2		3		4		5		6		7		8		9		10		
	Toc.*	Fat*	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.	Fat	Toc.		
1953																					
1	555	.....	225	.....	910	.....	990	.....	125	.....	1500	.....	70	.....	820	.....	275	.....	.....		608
2	5	.....	2090	.....	0	.....	145	.....	30	.....	1365	.....	425	.....	310	.....	75	.....	.....		494
3	25	.....	0	.....	5	.....	115	.....	80	.....	840	.....	90	.....	285	.....	145	.....	.....		176
4	45	.....	10	.....	315	.....	90	.....	15	.....	1710	.....	145	.....	1965	.....	265	.....	.....		507
8	50	.....	175	.....	265	.....	10	.....	45	.....	90	.....	115	.....	1335	.....	115	.....	.....		244
12	160	.....	155	.....	165	.....	135	.....	210	.....	114	.....	0	.....	395	.....	525	.....	.....		206
16	25	.....	175	.....	370	.....	105	.....	120	.....	30	.....	20	.....	10	.....	20	.....	.....		97
20	20	.....	25	.....	40	.....	35	.....	35	.....	170	.....	35	.....	40	.....	5	.....	.....		45
24	0	.....	70	.....	220	.....	65	.....	30	.....	35	.....	15	.....	50	.....	30	.....	.....		57
28	0	.....	35	.....	45	.....	35	.....	20	.....	10	.....	20	.....	80	.....	5	.....	.....		28
32	35	.....	245	.....	175	.....	25	.....	10	.....	50	.....	35	.....	55	.....	25	.....	.....		73
Av. colos.	158	.....	581	.....	308	.....	335	.....	62	.....	1354	.....	182	.....	845	.....	190	.....	.....		446
Av. milk	43	.....	126	.....	183	.....	58	.....	67	.....	71	.....	34	.....	281	.....	104	.....	.....		107
1954 **																					
1	63	0.63	493	2.47	123	1.60	322	0.37	68	0.30	165	0.63	421	3.20	710	3.44	248	2.13	63		268
2	77	0.80	869	4.10	414	3.67	25	0.57	774	3.00	160	1.20	32	0.67	63	0.67	35	0.63	137		259
3	414	2.27	192	2.13	239	3.23	23	0.93	68	3.06	42	1.17	35	0.93	1434	10.33	239	3.81	114		280
4	192	1.07	1696	7.94	331	2.23	32	1.00	65	1.37	134	1.97	488	3.67	105	2.37	165	1.63	12		322
8	72	0.97	17	1.87	77	0.97	192	0.80	192	3.33	2732	17.75	26	0.37	26	1.00	37	0.87	80		345
12	956	5.70	54	2.47	49	1.50	56	0.10	40	1.57	211	4.54	42	5.33	28	0.43	114	0.67	537		209
16	854	1.27	49	1.47	43	0.47	65	0.77	39	1.07	167	2.53	95	1.37	132	1.67	28	0.33	37		151
20	42	3.67	65	0.80	100	0.47	171	0.40	60	1.20	104	1.60	222	1.37	83	0.93	28	0.53	42		92
24	37	0.17	130	0.93	56	0.27	60	1.10	42	0.53	65	1.27	28	0.27	32	0.50	102	.....	28		58
28	32	1.17	65	0.43	51	0.37	32	.....	51	0.60	83	1.03	56	.....	37	.....	83	.....	56		55
32	42	.....	32	.....	46	.....	51	.....	51	.....	142	.....	65	.....	130	.....	.....	.....	.....		70
Av. colos.	186	.....	813	.....	277	.....	101	.....	244	.....	135	.....	244	.....	578	.....	172	.....	82		282
Av. milk	291	.....	60	.....	60	.....	90	.....	68	.....	531	.....	76	.....	67	.....	65	.....	130		140

* Tocopherol expressed as micrograms per 100 ml. of milk and fat as percentages.

** The cows sampled in 1954 are not the same individuals as those sampled in 1953.

of tocopherol per gram of fat, in 1954, were: 156, 81, 126, (herd 4 not sampled), and 409.

The literature values[1,3,9,11,12,15-20,22,24-27,29,37,39] for dairy cow milk tocopherol range from 20 to 150 μg. per ml., or from 9.6 to 54 μg. per gram of fat. Our dairy milk samples ranged from 24 to 150 μg. per 100 ml. Our Hereford milk samples, however, ranged in tocopherol from 0 to 1,804 μg. per 100 ml., or from 7 to 895 μg. per gram of fat. Individual cow averages varied from 16 to 501 μg. per 100 ml., or from 45 to 275 μg. per gram of fat. Herd milk tocopherol averages in 1953 were: 155, 40, 107, 240, and 17 μg. per 100 ml. In 1954 they were: 275, (herd 2 not sampled), 140, (herd 4 not sampled), and 40 μg. per 100 ml. Milk fat was determined only on herds 3 and 5 in 1954, with milk fat tocopherol levels of 130 and 48 μg. per gram of fat recorded.

It is thus evident that though the average values for the colostrum and milk toco-

TABLE 4—Tocopherol* Analyses of Milks from Herd 4

Days after calving	Cow number 1	2	3	Herd av.
		1953		
1	120	1120	585	608
2	60	1360	165	528
3	60	255	.....	158
4	40	160	.....	100
8	288	360	.....	324
32	204	110	.....	157
Av. colos.	70	724	375	390
Av. milk	246	235	.....	240

* Tocopherol expressed as micrograms per 100 ml. of milk.

TABLE 6—Tocopherol* Analyses of Milks from the Dairy Herd of the Montana State College

Days after calving	Cow number 298	295	93	286	187	Herd av.
			1953			
1	60	390	210	405	135	240
2	150	660	105	615	225	351
3	75	40	780	1100	65	412
4	90	85	105	330	40	130
8	140	65	150	85	45	97
12	55	100	60	75	25	63
16	40	60	140	80	30	70
20	85	105	50	40	395?	68
24	120	70	40	55	80	71
28	125	85	60	100	90	90
32	55	135	125	90	60	99
Av. colos.	94	294	300	612	116	283
Av. milk	88	88	89	75	55	79

* Tocopherol expressed as micrograms per 100 ml. of milk.

TABLE 5—Tocopherol and Fat Analyses of Milks from Herd 5

Days after calving	Cow number 1 Toc.*	1 Fat*	2 Toc.	2 Fat	3 Toc.	3 Fat	Toc. herd av.
			1953				
1	20	.....	.....	.....	.....	.....	.....
2	35	.....	.....	.....	.....	.....	.....
3	80	.....	.....	.....	.....	.....	.....
4	0	.....	.....	.....	.....	.....	.....
8	0	.....	.....	.....	.....	.....	.....
12	.....	.....	.....	.....	.....	.....	.....
16	20	.....	.....	.....	.....	.....	.....
20	10	.....	.....	.....	.....	.....	.....
24	10	.....	.....	.....	.....	.....	.....
28	5	.....	.....	.....	.....	.....	.....
32	55	.....	.....	.....	.....	.....	.....
Av. colos.	34	.....	.....	.....	.....	.....	.....
Av. milk	17	.....	.....	.....	.....	.....	.....
			1954 **				
1	44	0.53	574	0.50	234	2.50	284
2	382	4.17	325	0.23	345	2.13	351
3	502	1.07	630	1.87	641	0.67	591
4	40	0.71	77	1.53	40	0.90	52
8	17	0.87	31	0.67	31	1.33	26
12	40	1.53	49	1.10	35	1.20	41
16	35	1.17	40	0.70	31	0.67	35
20	46	0.73	56	0.67	42	0.70	48
24	106	1.17	51	1.43	37	0.53	65
28	28	.....	37	.....	37	.....	34
32	42	.....	32	.....	32	.....	35
Av. colos.	242	.....	402	.....	315	.....	319
Av. milk	45	.....	42	.....	35	.....	41

* Tocopherol expressed as micrograms per 100 ml. of milk and fat as percentages.
** The cows sampled in 1954 are not the same individuals as those sampled in 1953.

TABLE 7—Milk Fat Tocopherol* Levels

Days after calving	Herd 3 cow No.											Herd 2 cow No.		Herd 5 cow No.			Herd 1 cow No.					
	1	2	3	4	5	6	7	8	9	10	All	1	1	2	3	All	1	2	3	4	5	All
1	100	199	879	226	261	131	76	206	116	75	227	188	83	1148	93	441	403	242	161	541	123	294
2	96	211	43	258	133	47	112	94	55	42	109	23	91	1413	161	555	104	375	182	97	83	168
3	182	90	24	22	35	37	73	138	62	40	70	34	469	336	956	587	151	108	56	85	....	100
4	179	221	32	47	68	132	148	44	101	5	98	....	56	50	44	50	32	47	31	100	....	52
8	74	9	240	57	154	73	80	26	42	42	89	....	19	46	23	29	....	....	....	....	....	....
12	167	21	560	36	46	7	32	65	170	895	200	....	26	44	29	33	....	....	....	....	....	....
16	672	33	84	50	66	69	91	79	84	78	131	....	29	57	46	44	....	....	....	....	....	....
20	11	81	427	79	65	162	212	89	52	84	126	....	63	82	60	69	....	....	....	....	....	....
24	217	139	54	85	51	103	217	64	....	....	116	....	90	35	69	64	....	....	....	....	....	....
28	27	151	....	25	80	....	137	....	....	....	84	....	....	....	....	....	....	....	....	....	....	....
32	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
Av. colos.	139	180	242	138	124	87	102	120	84	40	126	81	175	737	314	409	172	193	108	206	103	156
Av. milk	195	72	268	55	77	83	126	65	87	275	130	....	45	53	45	48	....	....	....	....	....	....

* Tocopherol expressed as micrograms per gram of fat.

pherol levels from Hereford cows are similar to those reported for dairy cows, there are individual animals which deviate widely from these averages.

The manner in which the samples were collected was far from ideal. Since these are range cows which had never been closely confined, they were very wild. In order to obtain milk samples, they were driven into chutes and squeezes, where a few milliliters of milk were drawn from the frightened and struggling cows. It is possible that this procedure accounts for the enormous range of both fat and tocopherol encountered in individual samples. It is well known that the foremilk of dairy cows differs considerably in composition from the remainder of the milk and the strippings. That such differences include tocopherol concentrations was shown by Kieferle *et al.*,[15] who noted that the tocopherol level rose from 28 to 140 μg. per 100 ml., and the fat from 0.9 to 10.4 per cent during the course of a single milking.

The original plan called for the sampling of 10 cows from each herd, but in some cases it was not possible to milk that many animals. In spite of the possibility of sampling errors because of the small size of the samples, the data are presented here, with a full knowledge that some of the samples may not be representative of the herds from which they were taken.

In spite of the probable sampling errors, there may be significance in the great individual variations among cows in the same herd. Such variations indicate a need for additional fundamental research on the metabolism of tocopherols by lactating cows, particularly the factors which determine the movement of the tocopherols into the colostrum and milk.

The original purpose of this study, the correlation of colostrum and milk tocopherol levels of the cows with the occurrence of muscular dystrophy in the calves, was defeated by the failure of any calves to become dystrophic. However, it is evident that herd 2, which had experienced the greatest white muscle losses in previous years, had a much lower level of tocopherol in both colostrum and milk than had the other herds.

Another factor which must be considered in determining the tocopherol intake of the calves is the total volume of colostrum and milk ingested by them. We have

not attempted to make this measurement because of the practical difficulties involved in weighing the calves before and after each time they nurse. Though the dairy cow can store enough milk in her udder to raise a calf on four or five nursings a day, the beef calf must nurse more frequently to obtain its full milk ration, because of the limited storage volume in the udder of the beef breeds.

SUMMARY

1) Colostrum samples taken on the first, second, third, and fourth, and milk samples taken on the eighth, twelfth, sixteenth, twentieth, twenty-fourth, twenty-eighth, and thirty-second days after parturition from 52 semirange Hereford cows in five herds were analyzed for their tocopherol content. Corresponding samples from 5 dairy cows were similarly analyzed.

2) Averages of the tocopherol levels of the four colostrum samples from each beef cow varied from 18 to 1,537 μg. per 100 ml., or from 40 to 737 μg. per gram of fat.

3) Averages of the tocopherol levels of the seven milk samples from each beef cow varied from 16 to 501 μg. per 100 ml., or from 45 to 275 μg. per gram of fat.

4) Herd 2, with the lowest colostrum and milk tocopherol levels, was the herd which in previous years had suffered the greatest losses from white muscle disease (nutritional muscular dystrophy), though no cases of this disease were observed during the two years of this study, 1953 and 1954.

5) Colostrum and milk tocopherol levels from the 5 dairy cows were about the same as those of the beef cows, except for beef herd 2 which had much lower levels.

6) Definite evidence for the role of tocopherol deficiency in the etiology of naturally occurring white muscle disease could not be drawn from this study.

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Studies on Vaccination and Revaccination for Bovine Brucellosis

FRANCISCO SIEIRO, D.V.M.

Buenos Aires, Argentina

IN THE prevention of most livestock infectious diseases, it is generally understood that vaccinal antigens must be applied as nearly as possible to the time when infection most probably begins. In the case of seasonal diseases, it is often necessary to effect vaccination a few weeks before the outbreak can make its appearance. Such is the case in equine encephalomyelitis, anthrax, and other diseases against which vaccines generating a solid immunity can be used.

Prevention of bovine brucellosis has not been placed in this category. Despite its rather mild antigenic properties *Brucella abortus* strain 19 vaccine is applied early in life of the calves to prevent an infection which may occur eighteen to twenty months later. Vaccination closer to the critical time of receptivity is not advised, since postvaccinal agglutinins increase with sexual maturity.

Because of these limitations, various researchers have been looking for an improved method of immunization. Revaccination and adult vaccination have been assayed, the latter having been widely used in certain heavily infected areas. However, adult vaccination has been discouraged because of agglutinin interference in subsequent attempts of eradication.

As early as 1941, Haring and Traum [1] anticipated the possibility and convenience of revaccination as a means to enhance the immunity induced by calf vaccination, which they had already found decreasing by the second and third pregnancy.

Traum [2] reported good results in California revaccinating cows after calving. No data were recorded though little increase in the agglutinin response after the second vaccination was suggested.

In England, adult vaccination and revaccination have been extensively used. Lawson [3] reported on a field experiment with groups of animals vaccinated as calves; as calves and again as heifers; and as calves, heifers, and cows. Revaccinated animals yielded little or no better results than those with the single vaccination. Revaccination was done before or after parturition, the latter procedure seeming to be a common practice in that country.

Berman, Jones, and Beach [4] reported good results on the revaccination of a large herd in which the test and slaughter, and calf vaccination program, failed to eradicate the disease. Revaccination was performed within a month after calving, and the authors found that the agglutinin titers induced by the second vaccination showed a rapid decline.

Berman, Jones, and Irwin [5] were concerned as to whether the good results previously reported were attributable to revaccination or to the sanitary measures employed. In a new experiment, they vaccinated a group of calves 8 months old, revaccinating some at the age of 14 months and others at 20 months. All these animals, and a group of controls, were exposed during the third gestation period. There were no noticeably better results in revaccinated animals, whereas they showed a greater tendency to maintain suspicious or positive agglutination titers.

Calf vaccination with segregation or slaughtering of reactors has helped to eradicate or, at least, to minimize bovine brucellosis. However, sanitary measures are seldom applied save in the United States. In the majority of the countries which are at present using *Brucella abortus* strain 19 vaccine, prophylaxis is rather confusing; calf and adult vaccination, of healthy or even infected animals, often being indiscriminately practiced. Hence our desire to find a method which will give the cattle breeder maximum benefits from vaccination.

In this paper, a comparative immunity experiment on calf vaccination and revaccination is presented. In an attempt to shorten the time between immunization and exposure, one group of calves was vaccinated at the age of 10 months; a second group was vaccinated at 18 months; a third group of calves was vaccinated at 10 months and revaccinated at 18 months so that the second or "booster" inoculation would be given before the moment of greatest danger of contagion, that is, before the first pregnancy. All the animals and a group of unvaccinated controls were then subjected to exposure by conjunctival infection when approximately five to seven months pregnant.

MATERIALS AND METHODS

This test was done in the experimental farm of our Laboratory and the pathological, bacteriological, and serological investigations were performed

From the Instituto Rosenbusch S.A., Buenos Aires.

TABLE 1—Response of Vaccinated, Revaccinated, and Unvaccinated Pregnant Animals to Virulent Conjunctival Exposure

GROUP A¹—EARLY VACCINATED ANIMALS

N°	1952 8	9	10	11	12	1953 1	2	3	4	5	♀ 6	7	8	9	10	11	12	1954 1	2	3	4	5	6	B/I	Pregn. at Expos.	Date NB-AB	Post partum agglut	BACT. INVEST. fetus	placen	colost	Immun Classif
744		V																					E		6m	2/2 NB					IMMUNE
791		V																					E		6m 7d	2/2 NB					IMMUNE
801		V																					E		5m 25d	2/2 NB					IMMUNE
938		V																					E		6m 10d	2/2 NB					IMMUNE
005		V																					E		7m	2/2 AB		POSIT	POSIT	POSIT	INFECT
915		V																					E		5m 15d	2/2 AB		NEGAT		POSIT	INFECT
958		V																					E		6m 15d	2/2 AB		POSIT		POSIT	INFECT

GROUP B¹—TWICE VACCINATED ANIMALS

N°	1952 8	9	10	11	12	1953 1	2	3	4	5	♀ 6	7	8	9	10	11	12	1954 1	2	3	4	5	6	B/I	Pregn. at Expos.	Date NB-AB	Post partum agglut	BACT. INVEST. fetus	placen	colost	Immun Classif
675		V								R													E		7m 14d	2/2 NB					IMMUNE
783		V								R													E		5m 20d	2/2 NB					AB.RES
834		V								R													E		5m 20d	2/2 NB					IMMUNE
908		V								R													E		7m 7d	2/2 NB					IMMUNE

GROUP C¹—LATE VACCINATED ANIMALS

N°	1952 8	9	10	11	12	1953 1	2	3	4	5	♀ 6	7	8	9	10	11	12	1954 1	2	3	4	5	6	B/I	Pregn. at Expos.	Date NB-AB	Post partum agglut	BACT. INVEST. fetus	placen	colost	Immun Classif
016										V													E		6m 6d	2/2 NB				POSIT	AB.RES
035										V													E		5m 20d	2/2 NB				NEGAT	IMMUNE
079										V													E		5m 20d	2/2 NB				NEGAT	IMMUNE
088										V													E		6m	2/2 NB				NEGAT	IMMUNE
094										V													E		5m 8 d	2/2 NB				NEGAT	IMMUNE
37										V													E		5m 15d	2/2 NB				NEGAT	IMMUNE
63										V													E		6m 7 d	2/2 NB				NEGAT	IMMUNE
123										V													E		6m	2/2 NB				NEGAT	IMMUNE
187										V													E		6m	2/2 NB				NEGAT	IMMUNE
979										V													E		6m	2/2 NB				POSIT	AB.RES
593										V													E		5m 10d	2/2 NB				NEGAT	IMMUNE

GROUP D¹—UNVACCINATED CONTROLS

N°	1953 4	5	6	7	8	9	10	11	12	♀ 1954 1	2	3	4	5	6	B/I	Pregn. at Expos.	Date NB-AB	Post partum agglut	BACT. INVEST. fetus	placen	colost	Immun Classif
033															E		6m	2/2 NB				NEGAT	IMMUNE
060															E		6m 10d	2/2 NB				POSIT	AB.RES
073															E		5m 25d	2/2 NB				NEGAT	IMMUNE
45															E		7m	2/2 NB				POSIT	AB.RES
99															E		6m	2/2 NB				POSIT	AB.RES
232															E		7m	2/2 NB				POSIT	AB.RES
891															E		5m 18d	2/2 NB				NEGAT	IMMUNE
46															E		7m 10d	2/2 AB				POSIT	INFECT
233															E		7m	2/2 AB		POSIT		POSIT	INFECT
746															E		5m 18d	2/2 AB		POSIT		NEGAT	INFECT
977															E		4m 15d	2/2 AB		POSIT	POSIT	POSIT	INFECT

= Posit. Agglut. Titre 1/100 or Higher = Posit. Agglut. Titre 1/25

= Posit. Agglut. Titre 1/50 = Negative Agglutination

V—vaccinated; O—breeding time; E—1.5×10^{-8} conjunctival exposure; NB—normal birth; A—abortion.

in the Biological Division of the same. Forty-five grade Shorthorn or Argentine Holstein-Friesian calves, 8 to 9 months of age, were selected in August, 1952, and tested for Brucella agglutinins. When all proved negative, they were divided into three separate groups which were treated as follows.

Group "A," Early Vaccinated.—Fifteen calves, 10 months old, were vaccinated once subcutaneously on Sept. 7, 1952, with 5 cc. of a vaccine prepared with strain 19, containing 10 billion viable organisms per cubic centimeter.

Group "B," Twice Vaccinated.—Fifteen calves were vaccinated subcutaneously on Sept. 7, 1952, with the same vaccine and dose as those of group "A." On May 11, 1953, when 18 months old, they were revaccinated in a similar way, using a freshly prepared strain 19 vaccine of the same concentration.

Group "C," Late Vaccinated.—Fifteen calves were vaccinated once subcutaneously on May 11, 1953, with 5 cc. of the same vaccine, when approximately 18 months old.

Group "D."—Another 15 calves, negative to the blood test, were introduced to the farm in April, 1953, remaining as unvaccinated controls.

Until vaccinations were fully accomplished, groups A, B, and C were kept in separate lots and the unvaccinated controls were kept alone. After exposure, all the animals were gathered in a single unit.

Breeding by natural service started early in December, 1953, when bulls negative to the blood test were placed with each group of heifers. Unfortunately the experiment suffered a considerable reduction at this time as several heifers had been inadvertently bred by a bull not included in the trial. After the pregnant animals had been segregated, the number remaining in each group was as follows: Group "A," 7 animals; Group "B," 4; Group "C," 11; and Group "D," 11. On June 27, 1954, the vaccinated and control animals received a conjunctival exposure of 1,500,000 organisms of the virulent *Brucella abortus* strain 2308. Their pregnancies ranged from four and one half to seven months.

Throughout the whole experiment, blood tests were performed at monthly intervals by the Wright's tube method. After delivery, each of the cows again was tested three times to establish their postpartum agglutinin status.

After each parturition or abortion, appropriate cultures of various organs of fetuses and placental materials were made. Cultures of vaginal swabs were also tried, but this procedure was discarded because of frequent contamination and relatively poor results. Guinea pigs were inoculated with samples of colostrum of each cow and were killed six to eight weeks later. Cultures of various organs were made.

EXPERIMENTAL RESULTS

The details and the experimental results of this work are shown in table 1.

Cows of group "A" were exposed when pregnant five and one half to seven months (mean pregnancy stage 186 days). Four cows gave normal births without recovery of the infective organism, nor a rise in postpartum agglutination titers. Three cows aborted and were proved infected in subsequent bacteriological tests.

In group "B" the pregnancy state at the time of exposure ranged from five months and twenty days to seven months and fourteen days (mean pregnancy 195 days). The 4 animals calved normally without recovery of the organism but cow 783, which had maintained a low titer (1:25), increased this figure in the postpartum test, thus revealing infection.

The animals of group "C" were exposed when pregnant five months and eight days to six months and seven days (mean pregnancy 174 days). All of the 11 animals had normal births but 2 cows (016 and 979) revealed Brucella infection in the colostrum. The other 9 proved negative, though 4 of them showed low postpartum agglutination titers.

Unvaccinated controls (group "D") were exposed at pregnancies of four months and fifteen days to seven months and ten days (mean pregnancy 186 days). Three of the 11 gave normal births and proved negative in the bacteriological and serological investigations. Four others calved normally but eliminated the organism through the colostrum and had postpartum agglutination titers higher than 1:100. The other 4 cows aborted, showing infection and high agglutination values.

DISCUSSION

Following the methods of analysis used in a previous paper [6] the cows which, after exposure, calved normally and did not acquire infection traceable by bacteriological or serological methods were considered immune. Cows which aborted were considered infected as long as the virulent organism could be recovered, or when a rise in agglutination titer was demonstrated. Cows which gave birth to living calves, but which proved infected by bacteriological finding or showed a rise in postpartum agglutination titers to 1:100 or more, were considered "infected-abortion-resistant."

According to this criterion, the experimental results were more favorable to the revaccinated animals and to those vaccinated at 18 months, than to those vacci-

TABLE 2—Comparative Reactions of Early Vaccinated, Revaccinated, and Late Vaccinated Pregnant Animals to Conjunctival Exposure of 1.5×10^{8} Virulent Organisms

GROUP	N°	NB	AB	IMMUNE	INFECTED	
					abortion resistant	aborted
A' EARLY VACCINATED	7	4	3	4= 57,1 %	0	3= 42,8 %
B' TWICE VACCINATED	4	4	0	3= 75 %	1= 25 %	0
C' LATE VACCINATED	11	11	0	9= 81,8 %	2= 18,1 %	0
D' UNVACCINATED CONTR.	11	7	4	3= 27,2 %	4= 36,3 %	4= 36,3 %

NB—normal birth; AB—abortion.

nated at the age of 10 months. Both vaccinated and revaccinated animals gave better results than unvaccinated controls (table 2).

Data of this experiment, as far as revaccination is concerned, seem not to be in agreement with those obtained by other workers. Undoubtedly, the number of revaccinated animals in this test was too small for definite conclusions to be drawn. However, we believe that this subject is worthy of further experimentation since it apparently represents a method of reinforcing artificial immunity without too much interference with the prophylaxis, since agglutinins induced by the second inoculation are not very durable.

Similar recommendations can be made for late vaccination. This experiment confirms what we have observed in the field. For the last three years we have been advising this procedure in areas where no sanitary measures could be taken with excellent practical results.

Summary

A comparative study of the immunological values of early vaccination, of revaccination, and of late vaccination, in bovine brucellosis is reported.

One group of calves was vaccinated at 10 months of age, a second group at 10 months and again before breeding when 18 months of age; and a third group was vaccinated once at 18 months. Unvaccinated controls were added to the test. After exposure, all vaccinated animals showed less infection than unvaccinated controls, but the results were more favorable in the revaccinated and the late vaccinated groups, than in those vaccinated at the age of 10 months.

The results for late vaccination confirmed what has been observed by the author in field vaccinations.

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Cultivation of the Foot-and-Mouth Disease Virus in Explanted Epithelium of the Bovine Tongue
IX. Growth Characteristics of the Virus in Large Scale Tissue Cultures

H. S. FRENKEL and WILLIAM E. RIBELIN, D.V.M.

Amsterdam, Netherlands

THE LARGE scale cultivation of foot-and-mouth disease virus in tissue cultures consisting of bovine tongue epithelium has been described in previous publications.[1, 2] To date, preparation of vaccines from virus thus cultivated has been performed using culture material harvested at that period of incubation which limited tests had indicated as representing the highest infective titer within the culture. This has been at 20 hours of incubation. Little is known regarding the actual growth activities of the virus in tissue culture as well as the various environmental factors which may affect this growth. Information regarding virus multiplication in these cultures is necessary if the optimum value of such culture methods is to be achieved. Additionally, information regarding virus growth in tissue culture very likely will lead to a more complete understanding of the activities of the virus in the normally infected host animal. The experiments reported in this paper were designed to reveal the optimum time of culture harvest for maximum vaccine production, some of the factors affecting virus multiplication in large scale cultures and, lastly, to reveal as much information as possible regarding growth characteristics of the virus in such cultures. Application of the results reported herein to the virus growth processes in the host animal must be made with care. The experiments were designed primarily for application to vaccine production and, thus, are in many respects far from optimal conditions for precise studies on virus-normal host relationships. The converse is also true, as will be shown, in that the results of virus studies on tissues maintained in small, accurately controlled, flask or roller tube cultures may not always be applied unqualifiedly to large scale culture methods which are to be used for vaccine production.

Dr. Frenkel is director, The State Veterinary Virus Disease Research Institute, Amsterdam, Netherlands. Dr. Ribelin was formerly the research veterinarian, U. S. European Mission for Foot-and-Mouth Disease, Amsterdam, Netherlands. Present address: Department of Veterinary Science, University of Wisconsin, Madison.

MATERIALS AND METHODS

The results reported herein represent 17 experiments. The method of study for several experiments will be described; however, final results will represent a combination of all experiments. The procedures were as follows: The experiments were designed to study only a limited portion of the growth cycle at one time, generally 8 to 12 hours. This was necessitated by the great number of cattle necessary for infectivity titrations and the limited number of quarters available for animals. For the preliminary experiments, a series of flask cultures was prepared, each having the following composition:

Bovine tongue epithelium	15 Gm.
Baker's tissue culture medium, modified	150 ml.
Type O, Holland strain, virus	15 ml.
Penicillin	10,000 units
Dihydrostreptomycin sulphate	50 mg.

The cultures were prepared in 750-ml. Jena flasks stoppered in a manner which allowed continuous slow aeration with a mixture consisting of 95 per cent oxygen and 5 per cent carbon dioxide. The cultures were incubated at 37 C. At intervals of one-half to two hours over a period of eight to 12 hours, two flasks were removed and their contents pooled. The pH of the culture fluid was determined with a Beckman pH meter. Stained smears of the fluid were examined for bacterial contaminants.

The pooled cultures were ground by hand in a sterile mortar using quartz as an abrasive. The finely ground suspension was centrifuged for 15 minutes at 4,500 r.p.m. in a bucket-type centrifuge and the resulting supernatant was passed through Seitz E. K. filters at 0.3 kg. per square centimeter of pressure. The filters were moistened with tissue culture mediums prior to use. The resulting filtrates were stored at 5 C. throughout the duration of the experiment. The following day each sample was titrated by the intradermal lingual method in a single susceptible imported Irish ox using the method of Henderson.[3] Two tenfold dilutions in phosphate buffer, pH 7.6, were used per

ox, thus allowing ten observation sites per dilution. The dilutions used were those which would fall both above and below the anticipated 50 per cent infectivity point. These were generally in the neighborhood of 10^{-5} and 10^{-6}.

The results were plotted on semilogarithmic paper and the 50 per cent end point calculated by interpolation. Concurrent with these titrations, portions of each sample were titrated for complement-fixing antigen[4] using specific guinea pig hyperimmune type O (Holland strain) serum, sheep red blood cells and rabbit serum amboceptor, and guinea pig complement. Using a Fisher photoelectrometer, the complement was titrated in the presence of constant amounts of antigen and normal inactivated guinea pig serum, as well as alone, in order to determine the quantity of complement necessary to produce 50 per cent hemolysis of a given red cell suspension. The quantity necessary in the absence of serum-antigen was taken as the "unit." This quantity was then divided into the quantity required in the presence of antigen and antiserum, and the results were expressed as the units of complement fixed by the antigen. The results of these two types of titrations for each sample were plotted graphically and comparisons made.

Following these initial experiments, the work was repeated using large-scale cultures of the type utilized for vaccine production. Each culture was of the following composition:

Epithelium of 40 ox tongues	
Baker's medium, modified	14 liters
Type O virus, Holland, tissue culture origin	1 liter
Penicillin	1,000,000 units
Dihydrostreptomycin sulphate	0.5 Gm.
Chloromycetin	1.5 Gm.

The culture components were placed in a 60-liter stainless steel culture tank and incubated at 37 C. with aeration as previously described. For large scale studies, only one such culture was prepared for each experiment. At the appropriate intervals of time, the tip of a 100-ml. graduated pipette was introduced through a small corked orifice in the lid of the tank, two withdrawals totalling 200 ml. of the tissue-medium suspension were made, and incubation of the large culture was resumed. The sample was immediately centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant (medium) and sedimented (tissue) components were separated. One hundred milliliters of the supernatant was combined with 10 Gm. of the sediment, thus giving the same proportion as encountered in normal large-scale cultures when harvested for vaccine production. The mixtures were then ground, filtered, and titrated as described for the small flask cultures. However, 2 oxen were used for each sample in infectivity titrations, thus allowing 20 observation sites per dilution. Frequently these titrations were supplemented with infectivity titrations in unweaned white mice, a method which was developed as these experiments were reaching their climax. It has been shown to correlate quite closely with ox titrations[5,6] and its earlier availability would have permitted more extensive sampling.

In addition to these titrations, the samples from cultures incubated for longer than 20 hours were used for the preparation of vaccines in the following manner: In a number of vaccine bottles were placed 75 ml. of aluminum hydroxide and 1.5 ml. of glycocol (pH 9). The mixtures were autoclaved and allowed to cool. To each was added 75 ml. of the culture samples described in the preceding paragraph. These were incubated for 1 hour at 25 C. during which time they were frequently shaken. To each was added 0.75 ml. of 10 per cent formalin and all were incubated an additional 24 hours, again with frequent agitation. The vaccines were stored at 5 C. until used, at which time they were inoculated subcutaneously in doses of 1, 2.5, and 4 ml. into 15 guinea pigs per dose or 45 guinea pigs per culture sample. Four additional guinea pigs were inoculated into the rear foot pads to insure the absence of any infectious virus. Twenty-one days later each animal was challenged by inoculation into the right rear pad with 100 guinea pig infectious doses $(i.d.)_{50}$ of guinea pig-adapted type O virus originally derived from tissue culture. Four additional guinea pigs were vaccinated with 4 ml. of regular tissue culture vaccine and challenged later in order to verify specificity of the challenge virus. All guinea pigs were observed for protection against generalization of the virus and only those which displayed a large primary vesicle at the site of challenge were included in the vaccinal calculations. The numbers of animals protected with each dose were determined and the 50 per cent protective dose was calculated by plotting the percentage of animals protected as a linear regression of the vaccine dosage.[7] The protective doses thus represent an inverse indication of the amount of immunizing antigen present at each period of harvest.

Results and Discussion

The results of a typical experiment (No. 14) are given (table 1). Although the infectivity level drops after the 20-hour peak, the amount of immunizing antigen and the amount of complement-fixing antigen both continue to increase. In two other experiments, a decrease in infectivity and an increase in protective antigen were substantiated; in one of these, however, the complement-fixing antigen dropped steadily following the 20-hour peak and, in the other, the complement-fixing titer remained at the 20-hour level throughout the continuation of the incubation. This emphasizes the variability of

the complement-fixing antigen as an index of protective power.

In experiments involving the earlier hours of incubation, the following characteristics were observed. The titer of the cultures dropped rather rapidly following initial preparation, reaching a low within a half hour. The first increase in infectivity was detected between the second and fourth hours, this generally represented only a threefold or fourfold increase. Following this, the titer dropped somewhat to rise again about the sixth to eighth hour. Generally one or two further increases occurred until the peak was reached. The actual peak of infectivity varied widely, from 16 to 22 hours, with a mean of 19.2 hours. Degeneration of virus was rapid following the peak of infectivity with generally only one further small "release" occurring. No increases were ever detected after the thirty-eighth hour of incubation.

TABLE 1—Results of Titration of Tissue Cultured Foot-and-Mouth Disease Virus for Complement Fixation (A), Immunogenicity (B), and Suckling Mouse Lethality (C)

(A) COMPLEMENT TITRATIONS

Culture incubation (hr.)	Amount (ml.) 5% complement for 50% hemolysis	Complement fixed (units)
16	0.337	1.8
20	0.432	2.3
24	0.377	2.0
38	0.485	2.58
48	0.575	3.06
63	0.672	3.58

(B) VACCINAL TITRATIONS (GUINEA PIGS)

Culture incubation (hr.)	Vaccinal dose (ml.)	No. anim. protected/ No. anim. challenged	Protected (%)	Protective dose$_{50}$ (ml.)
20	1.0	5/14	35.7	
	2.5	8/14	57.1	1.95
	4.0	11/13	84.6	
28	1.0	6/13	46.1	
	2.5	7/14	50.0	1.85
	4.0	9/13	69.2	
48	1.0	6/15	40.0	
	2.5	10/15	66.6	1.60
	4.0	10/12	83.3	
63	1.0	13/15	86.6	
	2.5	10/11	90.0	<1.00
	4.0	14/14	100.0	

(C) INFECTIVITY TITRATIONS (UNWEANED MICE)

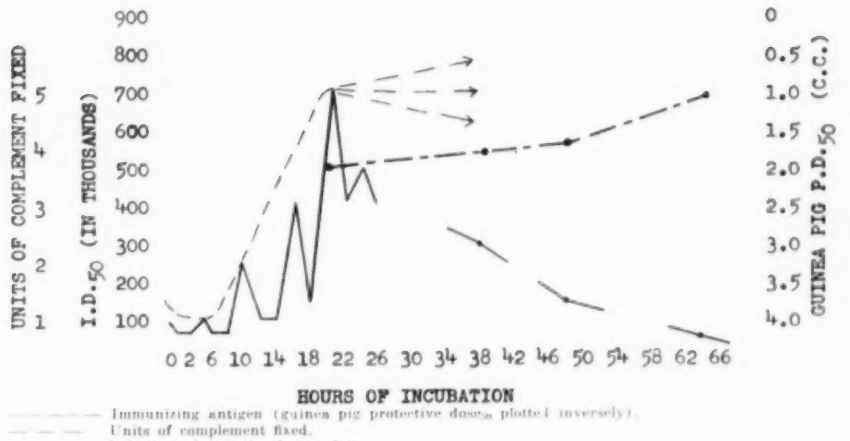
Culture incubation (hr.)	Dilution of inoculum	No. deaths/ No. inoculated	Calculated lethal dose$_{50}$
16	10^{-5}	11/12	434,000
	10^{-6}	4/15	
	10^{-7}	0/10	
20	10^{-5}	13/19	731,000
	10^{-6}	6/13	
	10^{-7}	0/9	
24	10^{-5}	14/15	325,000
	10^{-6}	1/11	
	10^{-7}	0/10	
38	10^{-4}	10/10	316,000
	10^{-5}	9/12	
	10^{-6}	3/12	
	10^{-7}	0/9	
48	10^{-4}	12/12	154,000
	10^{-5}	8/13	
	10^{-6}	0/8	
63	10^{-4}	10/10	158,000
	10^{-5}	10/16	
	10^{-6}	0/8	

Graph 1—pH changes at different periods of culture incubation.

Complement-fixing titrations roughly followed the infectivity curve; however, the former test is much less sensitive than the latter and increased in a gradual upward curve, showing the stepwise effect only when the virus increases were of great magnitude. Upon reaching the peak as indicated by infectivity tests, the complement-fixing titer was extremely variable, sometimes rising, sometimes falling, sometimes remaining stationary.

Guinea pig protective tests tended to support previous reports that the protective power of foot-and-mouth disease vaccine was not in accordance with the infective virus content. Thus, Fogedby and Johnson [8] reported good immunizing properties of cultures incubated so long as to contain no demonstrable active virus, and Möhlmann [9] stated that the peak of immunizing material in foot-and-mouth vesicles of the bovine tongue appeared after the occurrence of the highest demonstrable infective titer.

The varying occurrence of the peak of infective virus poses a problem in the selection of the optimum time of culture harvest. Workers with other viruses have


UNITS OF COMPLEMENT FIXED
I.D.50 (IN THOUSANDS)
GUINEA PIG P.D.50 (C.C.)
HOURS OF INCUBATION
0 2 6 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66
Immunizing antigen (guinea pig protective dose50 plotted inversely).
Units of complement fixed.
Infectious dose50 (in thousands).

Graph 2—Combination of all experiments—growth curve of foot-and-mouth disease virus in large-scale tissue cultures.

shown that the peak occurrence of virus growth is related, among other factors, to the size of the original inoculum.[10,11] To test this factor as a possible factor in the varying peaks which were encountered, two experiments were devised wherein a series of cultures were prepared and inoculated in the usual manner; a second series of cultures was inoculated with the same seed virus diluted 1:10 in phosphate buffer (pH 7.6). The cultures were incubated in the usual manner and paired cultures harvested during the period of 16 to 26 hours of incubation. As would be expected, the culture receiving the larger inoculum reached its peak at 20 hours, the culture receiving the lesser inoculum at 24 hours. The final titer of the latter cultures closely approximated that of the former cultures but did not quite equal it. It would not be surprising if more extensive trials revealed that the final titers of both would average about the same.

Experiments involving the earlier hours of incubation have revealed a considerable variation in the time required for different cultures to complete their eclipse phase and display the first virus increase. This, in turn, undoubtedly is reflected in the variable times of the occurrence of the growth peaks. This variation in initial growth has been shown to be due to the differences in time required for the temperature of the mediums and tissues to be brought to 37 C. The more rapidly the temperature is reached, the more rapidly does growth commence.

The pH levels of cultures harvested in series throughout the half-hour to 62-hour period of incubation are given (graph 1). The fact that foot-and-mouth disease virus apparently will not multiply when suspended in fluids of less than pH 6.9[12] probably accounts, in part, for the rapid cessation of growth and the inactivation of virus in cultures incubated for long periods of time.

The combined impressions from all experiments are depicted (graph 2) as representing the hypothetical growth curve of foot-and-mouth disease virus in large scale tissue cultures.

Summary

Foot-and-mouth disease virus (type O, Holland strain) multiplies in large scale tissue cultures in a stepwise manner as do most viruses in tissue culture. Titrations of the whole culture revealed the first detectable virus increase as occurring at about the fourth hour of incubation. Following a drop in titer, a second increase occurred about the eighth hour, being followed by one or two further increases until the peak was reached. After reaching the peak, one or two lesser increases occurred. No increases were ever detected after the thirty-eighth hour. The peak of infectious

virus occurred at various times between the sixteenth and twenty-second hours with a mean of 19.2 hours. The rapidity with which the peak of growth was reached was proportionate to the size of the inoculating dose.

Titrations of immunizing capacity indicated that this property continued to increase beyond the time when the peak of infectivity was reached. The longest period studied was 63 hours, at which time the immunizing titer was still rising, though infectious virus had almost disappeared.

Titers of complement-fixing antigen roughly followed those of infectivity titers; however, the former test was less sensitive and, thus, was often unable to detect the stepwise increase but showed merely a gradual upward rise. The peaks of infectious and complement-fixing titer generally coincided; however, the relationship was highly variable thereafter. In some cases the complement-fixing titer continued to rise; in others, a gradual decrease occurred. Thus, a continuous relationship between complement-fixing and immunizing components appears to be questionable.

ADDENDUM

Following preparation of this manuscript for publication our attention was drawn to a recent article by Ubertini, B., *et al.* (*Vet. Ital., 6,* [1955]: 403–418) on similar aspects of this problem. The experimental details were not given but the authors concurred with the findings reported herein that the growth curve of infectious virus is not paralleled by the changes in immunizing antigen. These workers recommended, however, the use of complement fixation as a means of determining the level of immunizing antigen. The reason for the latter discrepancy with our findings can not be determined from the information given in the article.

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Adenocarcinoma of the Uterus of the Cow—Differentiation of Its Pulmonary Metastases from Primary Lung Tumors

A. W. MONLUX, D.V.M., Ph.D.; W. A. ANDERSON, D.V.M., M.S.;
C. L. DAVIS, D.V.M.; W. S. MONLUX, D.V.M., Ph.D.

Denver, Colorado, and Ames, Iowa

VIRCHOW'S observation in the nineteenth century that organs to which epithelial tumors metastasize are rarely the site of primary carcinoma is still a fundamental to be considered in the study of metastatic carcinomas. Today, if Virchow could review the expanding literature on the pathogenesis of malignant tumors, he would probably agree that his postulation is not as axiomatic as it appeared to be in the infancy of cancer research. The increased recognition or occurrence of primary pulmonary neoplasms in man would undoubtedly amaze him. He would also be surprised to find that the majority of the primary lung tumors diagnosed in man have been proved to be derived from epithelial cells and that many of these are diagnosed as carcinomas.

In recent years in the United States, there has been an increasing tendency among veterinary pathologists to regard an appreciable number of the metastatic adenocarcinomas of cattle which have neoplastic lesions in the lungs and other variable anatomic sites as primary pulmonary tumors. Many of these tumors have been found by practitioners and meat inspectors. They have usually noted massive involvement of the lungs and scattered lesions in the remainder of the viscera and have submitted specimens to laboratories in the hope that the primary site might be apparent in histological studies. There have been many requests for detailed information about this group of tumors.

In 1951, a survey was conducted of all cases of neoplastic disease of food-producing animals in the files at the Animal Disease Research Laboratory at Denver, Colo. In older cattle, cases of metastatic adenocarcinoma with neoplastic involvement of the lungs and lymph nodes and sometimes other visceral organs were frequently encountered. In a review of histological preparations and available reports of these metastatic carcinomas, a pulmonary origin could be established only in a minimal number of the cases. Distinctive tubular structures, such as neoplastic bile ducts and uniferous tubules, suggested a definite primary site in some of the metastatic carcinomas. In others, the primary tumor had been included and was recognizable in the submitted tissues. Of the remaining metastatic bovine carcinomas, the authors were impressed with the similarity in the microscopic appearance and the necropsy findings of a large group of scirrhous adenocarcinomas in which the lesions were indistinguishable from metastatic lesions seen in cases of uterine carcinomas. There was no distinct resemblance of this group of tumors to any other common metastatic carcinomas. A tentative diagnosis of probable tubular genital tract tumors was made for this group of metastatic scirrhous carcinomas. In this survey, only five cases of primary lung tumors were recognized and four of these were represented by multiple lung lesions with no extrapulmonic metastases.

After this survey was completed, an agreement was made with the Federal Meat Inspection Service at Denver whereby veterinarians from the Animal Disease Research Laboratory cooperated in conducting and evaluating detailed necropsies of cases of neoplastic disease found at postmortem inspection. In the resulting studies, comparatively small, single or multiple lesions of an adenocarcinoma of the uterus with extensive metastases to the lung, visceral lymph nodes, and organs of the abdominal cavity were found in a significant number of affected animals. During the year 1953, nine of the 26 cases of adenocarcinomas of the uterus reported in this article were found. This increased recognition of uterine carcinoma does not imply a higher occurrence of the tumor in recent years. Actually, the recovery and identification of diseased bovine uteri containing small primary tumors is difficult in most abattoirs due to the accepted practice of discarding these organs after a cursory inspection for gross abnormalities. Obvious objections toward placing these organs into either pan of the standard paunch truck have necessitated such a procedure. (A few large meat-packing establishments now have installed movable pans on a chain assembly for the viscera inspection of cattle; this allows a routine inspection of the genitourinary organs with the re-

From the Animal Disease Research Laboratory (A. W. Monlux, Anderson, and Davis), Agricultural Research Service, U.S.D.A., Denver Federal Center, Denver, Colo., and Department of Pathology (W. S. Monlux), Division of Veterinary Medicine, Iowa State College, Ames.

We are indebted to Dr. J. W. Hovorka, inspector in charge, Dr. A. L. Stigers, assistant inspector in charge (now inspector in charge, Meat Inspection Branch, ARS, U.S.D.A. at Dubuque, Iowa), and to the veterinarians assigned to the Meat Inspection Branch, ARS, U.S.D.A., Denver, for their cooperation with veterinarians at the Animal Disease Research Laboratory in the collection of specimens. Detailed necropsies of many of the generalized cases of adenocarcinoma of the uterus mentioned in this paper were possible under this arrangement.

mainder of the viscera.) In many instances, small uterine lesions are recovered and made available for detailed study only through the alertness of the veterinarian conducting the inspection.

This cooperative tumor project has been gratifying from an academic viewpoint in respect to the metastatic adenocarcinomas, as primary sites in the uterus, kidney, ovary, or other organs could usually be established when the entire viscera and carcass were available for examination. Also, a group of four carcinomas considered primary in the lung parenchyma with thoracic or cervical lymph node metastases or both have been recognized. With the five pulmonary neoplasms reviewed in the preliminary survey, they made a total of nine primary epithelial lung tumors available for this study. While this group is small and admittedly incomplete from a differential standpoint, they are discussed because of the need to show how they differ from metastatic pulmonary adenocarcinomas. The controversy about the diagnosis of lung tumors in any species and the few reported cases of primary pulmonary neoplasms in the bovine species make their presentation of interest. Recognition of the more common primary sites of metastatic bovine carcinomas and the appearance of their metastases were most helpful in the evaluation of cases presented in this paper. It indicated that most of the large group of metastatic adenocarcinomas, suspected to have had their origin in the tubular portions of the genital tract in the 1951 survey, had their primary sites in the uterus. Pertinent data relative to these cases and to cases with metastatic lesions of probable uterine carcinoma diagnosed since the preliminary investigation are summarized (tables 3 and 4). The occurrence of this type of tumor in female animals only is essential supporting evidence for a diagnosis of probable uterine carcinoma. However, in "the cancer age" or older age group of cattle, a far greater number of cows are slaughtered than the combined totals for bulls and steers.

The gross and microscopic differential diagnosis of these uterine tumors from various granulomatous and parasitic conditions and from other neoplastic diseases (except for the data on primary lung tumors) is considered only in generalities in this paper because of the desirability of narrowing the discussion to a consideration of uterine and lung tumors.

LITERATURE CITED

Joest,[13] in 1924, summarized in a very complete and judicious manner much of the European and American literature on adenocarcinoma of the uterus of the cow and emphasized the difference of opinion among investigators in regard to their incidence. Perhaps the earliest observation on a relatively large group of these adenocarcinomas was made by Guillebeau[8] in 1899 at Basel, Switzerland, and later summarized and enlarged by Scholer[25] in 1916. Guillebeau observed seven cases but believed five were carcinomas of the cervix. From his gross and microscopic descriptions and illustrations, it is probable that all were of uterine origin. Scholer found two additional cases, in cows 8 and 10 years old, at Basel in the inspection of 12,225 slaughtered cattle and suggested that all of Guillebeau's cases were also adenocarcinoma of the uterus. The most complete early observation was made in 1902 by Sticker[31] in Berlin. In his series of 78 primary carcinomas in cattle, he found 44 (56%) to be present in the urogenital tract. Of these 44, ten were uterine carcinomas. Joest[13] cites the work of Carnat at the Bern Veterinary Pathologic Institute from 1887 to 1918, who found carcinoma of the uterus in the cow in six of 131 tumors, and of Detroix, who found four "epitheliomas" of the uterus among 49 cattle tumors. Joest also mentioned Schutz and Johne's observation on 78 bovine cases of carcinoma in which 56.4 per cent involved the urogenital tract. Wyssmann,[35] Sedlmeier,[28] Karetta,[14] and Thieulin[33] are other European investigators who have described individual or limited numbers of bovine uterine adenocarcinomas.

Trotter's statistics[32] on neoplasia have been quoted frequently by those who suggest that adenocarcinoma of the uterus in cattle is rare. He observed tumors in 300 cattle, including 298 females, and diagnosed but one case of adenocarcinoma of the uterus (a case earlier reported by Murray[17]). Joest and others attempt to explain the discrepancies between Trotter's and Sticker's observations on the incidence of carcinoma of the bovine uterus by pointing out that Trotter's cases included relatively old animals (75% over 15 years of age), while Sticker's cases represented all age groups. In the obviously limited and somewhat disguised observations made by Loeb and Jobbson in 1899, 49 carcinomas in 2,514,446 cattle slaughtered in Chicago were observed. They described the cattle as consisting predominantly of cows over 6 years of age. The conclusion that carcinoma of the uterus was rare because they found none among the 49, and the thoroughness of their observations can rightfully be questioned when a perusal of Loeb's publication[16] reveals that 48 of the 49 cases were carcinomas of the eye or its appendages.

Ball and Boudet[1] reported an interesting case of a uterine carcinoma in 1925. A 6-year-old cow had been manually delivered of a normal calf. In three weeks, the cow was inappetent and constipated. Difficulty in urinating prompted an examination of the genital organs at the end of the fourth week. A large tumor was palpated in the uterus near the cervix as well as greatly enlarged pelvic and sublumbar lymph nodes. In a few more days, the animal became dyspneic. Massive uterine hemorrhages into the peritoneal cavity were responsible for her death 40 days after the dystocia. A microscopic examination confirmed the presence of a uterine carcinoma with metastases to the sublumbar lymph nodes and lungs.

The case reported by Udall, Fincher, and Cushing[35] in 1926 as cancer of the cervix of a cow could well have been an adenocarcinoma of the uterus in view of the histological diagnosis of carcinoma of the cervix and uterus. The cow, a 6-year-old Holstein-Friesian, progressively lost her condition and died after an observed illness of several months. Davis, Leeper, and Shelton,[3] in 1933 at Denver, Colorado, reported that four of 90 bovine tumors collected by them during a five-year period were adenocarcinomas of the uterus. The four adenocarcinomas had been found in federally inspected abattoirs but were not available for inclusion in the present study. Feldman reviewed and confirmed the diagnoses made by the group at Denver and used these cases and others for material for his monograph[7] and at least one other article[6] on neoplasia of the domesticated animals.

Ottosen's description of one bovine scirrhous adenocarcinoma of the uterus in 1941[21] and of 14 cases in 1943[22] are well documented. In eight of his cases, metastases were found in the lungs. Ottosen's first case was reported from Copenhagen, Denmark, and had been detected by a practicing veterinarian while doing a rectal examination on an older cow because of a history of retention of the fetal membranes, and sterility of several months' duration. Ottosen later continued his research in Stockholm, Sweden, at the Royal Veterinary College and the State Veterinary Medical Institute and the 14 cases included in his second report had been diagnosed at those institutions. Clinical signs were recorded for 3 of these animals; 1 had become sterile, a second with lung metastases had been noticeably ill and had loud râles over the lungs, and the third, in which extensive peritoneal lesions were found, had been treated for indigestion and recurring tympanitis. Ottosen mentioned the frequent recognition of scirrhous adenocarcinoma in the lungs of cattle (usually in combination with other carcinomatous metastatic lesions in lymph nodes and abdominal organs) and believed that most of them originated as metastases of primary tumors of the uterus. He stated that 47 cases of scirrhous adenocarcinoma of the lungs had been found at the Royal Veterinary College and the State Veterinary Medical Institute in Sweden in their investigations of neoplastic diseases. Ottosen also described Harbitz's work[9] at the Royal Veterinary College and the Veterinary Pathological-Anatomical Institute in Norway where Harbitz had observed three cases of carcinoma of the uterus and 37 cases of scirrhous adenocarcinoma of the lung. Ottosen believed that these 37 cases were similar to the 47 cases of scirrhous adenocarcinoma of the lung studied by him at Stockholm.

Lagerlof and Boyd[15] in Sweden made necropsy examinations of the sexual organs of 6,286 dairy cattle of the Swedish Highland breed during a two-year period ending in 1952 in a study of ovarian hypoplasia. One scirrhous adenocarcinoma of the uterus was an incidental finding.

Recently, Dobberstein,[4] in Germany, made a statistical evaluation of 1,241 bovine tumors which

TABLE 1—Bovine Uterine Adenocarcinomas and the Location of Their Recognized Metastases in Selected Anatomic Sites

Case No.	Estimated age (yr.)	Breed (when known)	Lung	Bronchial lymph node	Mediastinal lymph node	Sublumbar lymph node	Internal iliac lymph node	Ovary	Peritoneal surfaces
D 3281	old	.....	+	—	+	+	+	—	+
D 3196	aged	.....	—	—	—	—	—	—	+
D 3090	12	Hereford	—	—	+	+	+	+	+
D 2973	7	.....	+	+	+	—	—	—	—
D 2907	2	Hereford	+	—	—	+	+	—	—
D 2824	9	.....	+	+	—	+	+	—	+
D 2786	12	Hereford	+	—	—	+	+	—	+
D 2696	7	Hereford	+	+	+	+	+	—	+
D 2691	10	Hereford	+	+	+	+	+	+	+
D 2662	8	Hereford	+	+	+	+	+	+	—
D 2647	7	.....	+	+	+	—	—	—	—
D 2477	7	Hereford	+	—	+	—	+	—	+
D 2422	9	.....	+	—	—	—	+	—	—
D 2332	8	Hereford	+	+	+	+	+	+	+
D 2031	10	Hereford	+	+	—	—	+	—	—
D 2001	11	.....	+	+	+	+	+	—	—
D 1999	5	.....	—	—	—	—	—	—	—
D 1354	7	.....	+	+	+	—	—	—	+
D 1450	15	.....	+	—	—	+	—	+	—
D 1210	11	Hereford	+	+	+	+	—	—	—
D 810	10	.....	+	—	+	+	—	—	—
D 669	aged	Hereford	—	—	—	—	—	—	—
129472	old	.....	+	—	—	—	—	—	+
125820	4	Br. Swiss	+	+	+	+	+	—	+
15214	aged	.....	+	—	—	—	—	—	+
15063	4	.....	+	—	—	+	+	—	+
Totals			22	12	14	15	17	5	14

+ Location of a metastatic lesion; — no recognized metastasis.

had been reported by various investigators. He listed 393 cases of carcinoma and indicated that 37 of these were carcinomas of the uterus. His tables show only carcinomas of the eye, stomach, liver (and gall bladder), ovary, and skin with a higher reported incidence than uterine carcinomas. Dobberstein mentioned that, of the domesticated animals, the rabbit and cow develop uterine carcinomas most frequently. The occurrence of uterine carcinomas in the other domestic animals reported by Dobberstein can be summarized from his tables as: one of 156 tumors (73 carcinomas) in cats, four of 1,925 tumors (773 carcinomas) in dogs, one of 188 tumors (25 carcinomas) in swine, none of 114 tumors (36 carcinomas) in sheep, and six of 1,222 (546 carcinomas) in horses.

PATHOLOGICAL INTERPRETATIONS

The age, breed, and distribution of lesions in 26 cows with adenocarcinoma of the uterus are reported in this study (table 1) and the diagnoses of five other carcinomas which were regarded as primary tumors of other segments of the tubular portion of the bovine genital tract are given (table 2). The cases listed in these two tables represent all the examples of primary adenocarcinoma of the tubular portions of the bovine female genital tract diagnosed at the Denver laboratory. The tumors of the cervix recorded in table 2 resembled the uterine carcinomas in respect to pattern of metastasis and secondary tissue reaction and were of less importance only because of infrequent occurrence.

Only four of the 26 uterine tumors and two of the five tumors of the genital tubular organs, other than the uterus listed in tables 1 and 2, did not have lung metastases. The predominance of lung and lymph node metastases indicates that these cases of uterine carcinomas represent advanced stages of a malignant tumor. Observations can be made in regard to the distribution and appearance of the multiple lesions which should be helpful in the recognition and understanding of this disease when encountered clinically or at necropsy. Data from some of the early cases were meager, so some metastases may have been overlooked or not recorded. However, table 1 lists the recorded occurrence of metastases in lymph nodes which are closely related to the metastasis of the tumor from the uterus to the lung. The internal iliac lymph nodes, which drain the lymphatics from much of the female genital tract including the uterus, and the sublumbar lymph nodes are routinely examined in meat inspection to detect, in addition to other conditions, evidence of tumor metastases from the genitourinary organs. In animals with lung involvement, once metastases were present in these regional lymph nodes, several individual or combinations of routes of metastasis may have been followed to the lungs. Since the tumor often invades through the capsule of lymph nodes, through the walls of the lymphatics, or stays in the lymphatic channels, it could easily follow in or along existing lymphatics near the aorta and finally become established in many of the sublumbar lymph nodes. During this progressive lymphatic spread, there is ample opportunity for the tumor to enter the venous blood system, either by invasion of veins or directly through the lymphatic tributaries of veins. Embolic dissemination via the

TABLE 2—Bovine Carcinomas, Other than Uterine, of the Female Tubular Genital Tract and the Location of their Recognized Metastases in Selected Anatomic Sites

Case (No.)	Estimated age (yr.)	Breed (when known)	Diagnosis and primary site	Lung	Bronchial lymph nodes	Mediastinal lymph nodes	Sublumbar lymph nodes	Internal iliac lymph nodes	Ovary	Peritoneal surfaces
D 3150	8	Hereford	Squamous cell carcinoma of vulva	—	—	—	—	—	—	—
D 1743	6	.....	Squamous cell carcinoma of cervix	+	+	+	+	—	—	—
D 340	10	.....	Adenocarcinoma of cervix	+	+	+	+	+	—	—
127523	6	Hereford	Squamous cell carcinoma of vulva	—	—	—	—	—	—	—
17699	10	.....	Squamous cell carcinoma of vagina	+	—	—	—	—	—	+
Totals				3	2	2	2	1	0	1

+ Location of a metastatic lesion; — no recognized metastasis.

veins or thoracic duct certainly could produce the multiple lung lesions seen in our cases. It is quite probable that the main tributaries to the cisterna chyli and the thoracic duct or ducts* are frequently invaded directly; two cases in which large portions of the thoracic ducts were distended with tumor thrombi and emboli were available for detailed study. Arterial invasion was never recognized, even though involvement of veins and larger lymphatics was frequently evident as thrombi and emboli in vessels in the lungs, uterus, and mediastinum (fig. 25).

Further evaluation of table 1 reveals a large group of cases in which there were metastases to peritoneal surfaces. The omission in the table of notations regarding liver, spleen, kidney, and adrenal metastases, or other metastases to abdominal organs, is based on our belief that most of these lesions represent or originate from transperitoneal metastases and therefore can be grouped, to simplify their presentation, under metastases to a peritoneal surface. The necropsy findings in seven of the more recent cases with peritoneal metastases and the histological examination of tissues from all cases support this conclusion. Involved areas were consistently found where the tumor had made only a limited invasion of the subserous tissues of the involved organs or membranes. Most invasive liver lesions are believed to have their origin from transperitoneal metastases (fig. 7 and 26).

Lymphatic spread of the tumor to the serosa of the uterus, the fallopian tubes, and ovaries will be discussed in the section on microscopic data. These metastases and the early metastases to the regional and more distant lymph nodes would suggest that further extension through the subserous lymphatics to the peritoneal or abdominal organs could be expected. Transfallopian tube metastases of tumor fragments from a growth no longer retained by an intact endometrium or the rupture and liberation

* Two thoracic ducts were seen in some of the cattle examined in this study.

TABLE 3—Metastatic Adenocarcinomas in Cows Resembling Carcinomas of the Uterus with Submitted Lesions Limited to the Lungs or Lungs and Lymph Nodes Listed Below

Case (No.)*	Estimated age (yr.)	Breed (when known)	Bronchial lymph nodes	Mediastinal lymph nodes	Sublumbar lymph nodes	Internal iliac lymph nodes	Other lymph nodes
D 3384	5	.....	—	—	+	—	—
D 3348	5	.....	+	+	—	+	—
D 3297	8	.....	+	+	+	+	—
D 3280	10	.....	—	+	+	+	—
D 3269	9	.....	—	+	+	+	—
D 3054	5	.....	+	+	+	+	—
D 2914	old	Hereford	+	+	+	+	—
D 2834	8	.....	—	+	—	—	Cervical, prefemoral
D 2800	old	Hereford	+	+	+	—	Sacral
D 2765	6	Hereford	+	+	+	+	—
D 2763	5	Hereford	+	+	+	+	—
D 2719	7	.....	+	+	+	+	—
D 2685	old	Friesian Holstein-	+	+	+	+	Sternal, intercostal, deep inguinal, sacral
D 2655	8	Hereford	+	+	—	—	—
D 2594	4	.....	+	+	—	+	—
D 2348	5	.....	+	+	—	+	Posterior cervical, renal
D 2245	4	.....	+	+	+	+	Renal
D 2092	2	.....	—	+	—	—	Posterior cervical
D 1896	8	.....	+	+	+	+	—
D 1774	5	.....	—	—	—	—	—
D 1745	aged	.....	—	—	—	—	—
D 1739	5	.....	—	+	+	—	—
D 1718	4	.....	+	+	+	+	Renal
D 1669	aged	.....	—	+	—	—	Deep inguinal
D 1373	8	.....	+	+	+	+	—
D 1180	12	.....	—	+	+	+	Renal
D 1018	10	.....	+	+	—	—	—
D 1014	15	.....	+	—	—	+	—
D 843	old	.....	+	—	—	—	—
D 309	6	.....	+	+	+	—	—
16069	8	.....	—	+	—	—	—
15543	5	.....	+	+	—	—	Cervical, sternal
15343	6	.....	+	+	—	—	—
Totals			22	28	18	18	

* All cases were female animals; + location of metastatic lesion; — no recognized metastasis.

of tumor cells from tumefied lymph nodes or other diseased abdominal tissues are the only other logical source of peritoneal lesions. Palpation and manipulation of tumefied uteri, lymph nodes, or other abdominal tissues could actually hasten the dissemination of the tumor throughout the peritoneum by either the implant or lymphatic route. In the most recently examined case (D 3281), there were many small nodular growths on the peritoneum for a distance of about 3 to 4 cm. from one group of sublumbar lymph nodes. Lack of involvement of the remainder of the peritoneum suggested secondary metastases from lymph node to peritoneum.

In uterine carcinomas with lung metastases, general systemic metastases should not occur unless the capillary barrier of the lung is breached. Lack of evidence of systemic metastases in the brain, adrenal gland, kidney, skeletal muscle, and bone marrow supports our conviction that at least a very high percentage of certain questionable metastatic growths, particularly in the liver, did not have a systemic origin. It should be remembered, however, that the cows represented in our series were in sufficiently good condition to permit slaughter. Spectacular systemic metastases are usually found in cases of adenocarcinoma of the uterus in women only when there are symptoms suggestive of arterial embolism or in a few of the cases with histories of fatal metastasis. Perhaps, veterinary clinicians and pathologists will report an occasional systemic metastasis in the fatal cases of the disease which are brought to their attention. In the abdominal organs and particularly in the liver, we did observe the establishment in metastatic bovine uterine carcinomas of scattered, secondary satellite growths. It is possible that such growths could set up a cycle of metastasis independent of the primary uterine tumors. This secondary pat-

TABLE 4—Metastatic Adenocarcinomas in Cows Resembling Carcinomas of the Uterus with Submitted Lesions Establishing the Presence of Lung and Transperitoneal Metastases and the Lymph Node Metastases Listed Below

Case (No.)*	Estimated age (yr.)	Breed (when known)	Bronchial lymph nodes	Mediastinal lymph nodes	Sublumbar lymph nodes	Internal iliac lymph nodes	Other lymph nodes
D 3363	12	.....	—	—	—	—	Notation of "lymph nodes affected"
D 3298	6	.....	+	+	+	+	—
D 3239	8	Hereford	+	+	+	+	Sternal, posterior cervical
D 3208	7	.....	+	+	+	+	—
D 3107	old	Hereford	+	+	+	—	—
D 3068	7	.....	+	+	+	+	Renal, posterior cervical
D 3031	aged	Hereford	+	+	+	+	—
D 2843	8	.....	—	—	—	+	—
D 2719	7	.....	—	—	—	—	—
D 2574	5	Hereford	—	+	—	—	—
D 2176	6	.....	—	—	+	+	Sternal
D 2060	12	.....	—	+	—	+	Posterior cervical, mesenteric, prescapular, portal, popliteal
D 1742	6	.....	+	+	—	—	—
D 1539	6	.....	+	+	—	—	—
D 1348	mature	.....	+	+	+	+	Mesenteric, renal
D 1224	12	.....	—	+	—	—	—
D 308	6	.....	+	+	+	—	—
D 136	10	.....	—	+	—	—	—
17679	3	.....	—	—	—	—	—
17485	10	.....	—	—	—	—	Notation of "all visceral lymph nodes affected"
17121	adult	.....	—	—	—	—	—
15789	aged	.....	+	+	—	—	—
15664	8	.....	+	+	—	—	Portal, mesenteric
15497	7	.....	—	—	—	—	Notation of "thoracic and abdominal lymph nodes affected"
15433	7	.....	—	+	—	—	—
15141	6	.....	+	+	—	+	—
15094	5	.....	—	—	—	—	Notation of "body and visceral lymph nodes affected"
14897	8	Holstein-Friesian	+	+	+	+	Renal
14568	6	.....	+	+	—	—	—
Totals			15	20	10	11	

* All cases were female animals; + location of a metastatic lesion; — no recognized metastasis.

tern of metastasis would more closely resemble those of a malignant epithelial tumor of the invaded organ and could result in lymphatic spread to the posterior mediastinal lymph nodes. However, most mediastinal lymph node metastases in our cases were believed related to a lymphatic metastasis from within the thorax. Generalized metastases or death should soon follow the massive lung and abdominal cavity involvement present in many of our cases. Obviously, a stage is reached where the cow either becomes sterile or is in a noticeably poor physical state. The owner, if aware of the animal's condition, probably attempts either to institute medical treatment or sends the cow to market for her salvage value. On other farms and ranches where a careful animal husbandry is not practiced, the animals undoubtedly die.

Incomplete notations on the conditions of the cows (table 1) indicate that 18 were thin and in poor to fair condition at time of slaughter, 2 were in good condition (D 1210 and D 669), and 1 was a very fat animal (D 1450).

In the animals with metastatic adenocarcinoma indistinguishable from adenocarcinoma of the uterus (tables 3 and 4) some of the uteri were not available for a critical inspection. When the genital tract was examined in the remainder of these animals, no tissues or unsatisfactory specimens from a suspected uterine lesion had been forwarded for subsequent histological confirmation. A relatively large number of uterine tumors are probably encountered on postmortem inspection if a high percentage of these animals actually have adenocarcinomas of the uterus with metastases. The appearance of submitted lesions, pattern of metastasis, secondary tissue reaction, and occurrence in older cattle make them indistinguishable from our better documented cases of uterine carcinoma. The data (tables 3 and 4) were separated into two individual compilations so that it might better be correlated with the data and discussion of table 1. It is shown that 12 cases (table 1) of uterine adenocarcinoma had lung and peritoneal metastases while only lung or lung and lymph node metastases were recorded for ten other cases. Similar recorded metastatic lesions are listed for 29 cases and 33 cases, respectively, in tables 3 and 4. Ottosen [22] also had the conviction that many metastatic adenocarcinomas of the lung in cows are submitted for laboratory examination with the smaller primary uterine lesion being overlooked or not considered important.

As mentioned previously, the data presented in tables 3 and 4 represent only the cases in which the submitted lesions could not be differentiated from metastases of uterine carcinomas. While similarity does not constitute identity, we believe that diagnosis of probable uterine tumor is justified. If the idea of a primary lung tumor with abdominal metastases can be dispelled as being improbable, natural curiosity should immediately be aroused, in the more typical cases, as to the reason for the involvement of the sublumbar and internal iliac lymph nodes or the widespread peritoneal metastases. Only after the genitourinary organs and the posterior portions of the intestinal tract have been carefully examined for a primary tumor is there any justification for considering the possibility of a more remote and, hence, unusual site of origin. Future observations may produce evidence that other genitourinary organs are the probable primary sites of some of the tumors listed in tables 3 and 4. Kidney, ovarian, and adrenal tumors have been encountered in our studies of neoplastic disease but most of these are easily recognized and it is doubtful if there could be much confusion in diagnosis between tumors of those organs and adenocarcinoma of the uterus. As an example, the granulosa-cell tumor of the ovary has anatomic sites of metastasis in the peritoneal cavity and lymph nodes similar to a uterine carcinoma but is quite distinctive in its gross and microscopic appearance.

Some metastases were undoubtedly overlooked in the reports condensed into tables 3 and 4, although such a conjecture can not be applied to individual cases. We encountered the phenomenon of one or more lymph nodes being free of tumor while the neighboring nodes contained massive neoplastic deposits. In table 4, the lack of recorded metastases at some of the lymph node sites, particularly in earlier cases, can be qualified by the observation that postmortem notations submitted by contributors often gave detailed descriptions of lesions of the peritoneum or abdominal organs and only a limited or general statement in regard to lymph node metastases. Therefore, the lymph node lesions observed

undoubtedly exceeded the minimal recorded data shown in the table.

Five primary lesions of adenocarcinoma of the uterus are shown in the illustrations (fig. 1-6, 8, 9). Most of the tumors had their primary sites in the uterine horns with the left horn having about two-thirds of the lesions where notations of the exact locations of the growths were recorded. In a few instances, both horns were involved by a single tumor in the area where the muscular walls of both cornua blend together and have a common serosal covering. A primary site in the body of the uterus** could be established in only two cases (D 2691 and D 2422). The tendency for the lesions to form a sclerotic, annular constriction without perforation of the serosa and little or no extension onto the surface of the endometrium is its most consistent feature (fig. 1, 3, 5). These and the other types of uterine growths and their metastases were yellow to grey on section and characteristically quite fibrous or even nodular. Usually, the primary site is obvious.

One animal with three distinct growths in the uterine wall (two in one cornu and one in the other) is of interest.

Adenocarcinoma of the Uterus.—Necropsy of 9-Year-Old Cow, Case D 2824.—Three distinct, fibrous, grey to yellow growths were present in the walls of the uterine cornua. The largest of these growths (5 by 3 by 3 cm.) had produced a complete annular constriction of the right cornu with infoldings of its serosa about 15 cm. from the bifurcation of the horn from the body of the uterus. A second, smaller tumor (2 by 2 by 2 cm.) completely constricted the right cornu about 5 cm. cephalad from the larger tumor. The uterus between these neoplasms was filled with a watery, reddish fluid. The third growth (3 by 2 by 1 cm.) was found in the left cornu and was 10 cm. from the bifurcation of the horn from the body of the uterus. It had produced only a partial constriction of that horn. Uterine mucosa appeared to cover these growths within the uterus. Similar greyish tumor masses of varying sizes were seen in all lobes of the lungs and there was extensive scarring associated with growths. The main bronchi and their larger branches were free of neoplastic tissue. The tumor was additionally found on the omentum, peritoneum, mesenteries, broad ligament of the uterus, and on the serosa of the spleen and liver. Some of the hepatic growths were invading the parenchyma of that organ. Lymph node metastases were present in the bronchial, internal iliac, and sublumbar lymph nodes. No metastases were recognized in the mediastinal lymph nodes.

Lateral spread of one of the growths beneath intact endometrium is shown histologically (fig. 21 and 22).

One of the rather consistent findings in cows with extensive sublumbar and internal iliac lymph node metastases was nu-

** The designation of an anatomical body to the bovine uterus is actually erroneous as Williams[20] has pointed out. We use uterine body in this article to indicate an area 4 to 8 cm. long where the two cornua form a common cavity which is continuous with the cervival canal.

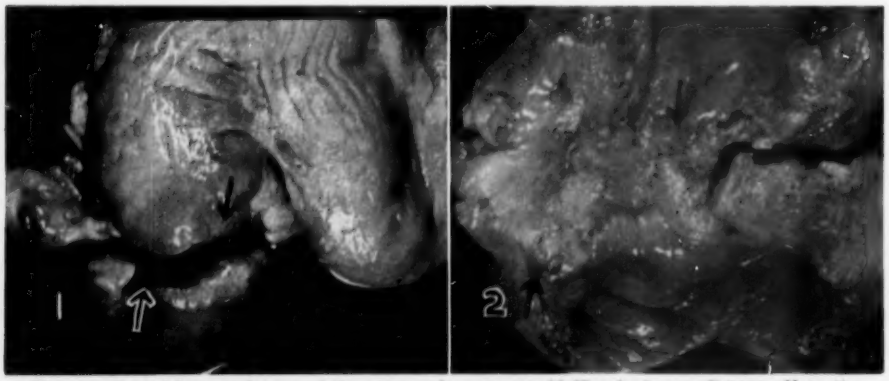


Fig. 1 and 2—Adenocarcinoma of the uterus of an 8-year-old Hereford, case D 2662. Note the partial annular constriction of the uterine horn (arrows).

merous thrombi in dilated, thin-walled vessels in the broad ligament and sublumbar region. Although a few of these vessels were proved to be veins by dissection techniques, many were undoubtedly distorted lymphatics. In two instances, the thrombi were also recognized in vessels in the mediastinal pleura (fig. 25).

Rectal, renal, and posterior cervical lymph node metastases were present in a few of the cows with adenocarcinoma of the uterus. In one of these animals with rectal lymph node involvement, there were also extensive metastases in the coccygeal vertebrae (D 2001).

Necropsy Findings in Selected Cases of Adenocarcinoma of the Uterus

Necropsy of 8-Year-Old Hereford Female, Case D 2662.—A firm, yellow, irregular shaped mass (6 by 5 by 3 cm.) was present in the middle portion of the right cornu of the uterus (fig. 1). This tumor had produced a partial annular constriction of the uterine horn (arrows) which still had a patent although tortuous lumen. The growth had eroded through the uterine endometrium and had replaced most of the uterine wall in the involved areas (fig. 2, the incised uterine lesion). A linear, cordlike growth extended from the main tumor in the submucosal tissues to, and along the entire length of, the fallopian tube and finally produced adhesions of the fimbria to the ovary. Many fibrous tumor nodules, up to 4 cm. in diameter, were found in all lobes of the lung. Tumor metastases were present in the bronchial, mediastinal, sublumbar, and iliac lymph nodes.

Necropsy of 8-Year-Old Hereford Female, Case D 2332.—The left cornu of the uterus contained a sclerotic tumor mass about 10 cm. from its junction with the body of the uterus (fig. 3). The neoplasm had produced a complete annular constriction (arrows) although it involved only about a 4 to 6 cm. length of the horn. The corresponding ovary (fig. 4, the incised tumefied [a] and nontumefied [b] ovaries, and uterine lesion) was enlarged and fibrous and contained definite masses of neoplastic tissue. Many small tumor nodules were present on the surface of the broad ligament adjacent to the affected cornu. The broad ligament also contained many thrombi in the utero-ovarian veins. Tumor masses, usually not more than 1 to 2 cm. in their greatest diameter, were scattered in the parenchyma of the lung and liver and as hundreds of nodules on the visceral and parietal peritoneum. A few tumor nodules were found on the parietal pleura. Large tumor metastases were present in the bronchial, iliac, and sublumbar lymph nodes and small tumefied areas were found in the portal and mediastinal lymph nodes.

Necropsy of 7-Year-Old Cow, Case D 1354.—Scattered tumor masses were present in the body and both horns of a greatly enlarged uterus. The cervix was not involved. Metastatic growths were present in both lungs, the liver, and the bronchial, mediastinal and internal iliac lymph nodes (*see* fig. 8).

Adenocarcinoma of the Uterus with Metastases.—Necropsy of 12-Year-Old Hereford Female, Case D 2786.—The animal was in poor condition. A yellow, fibrous growth (28 by 20 by 5 mm.) had produced a constriction of the dorsal wall of the left cornu of the uterus about 5 cm. from its bifurcation from the body, causing a de-

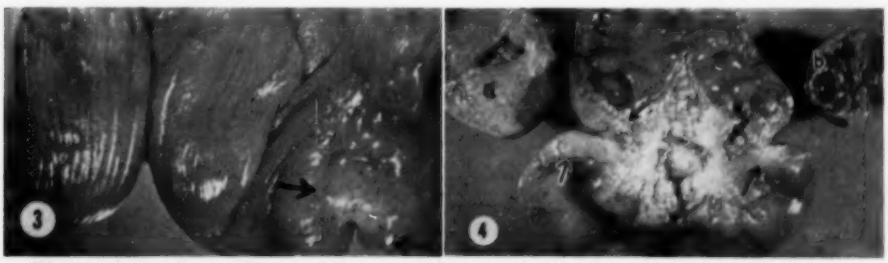


Fig. 3 and 4—Adenocarcinoma of the uterus of an 8-year-old Hereford, case D 2332. Note the complete annular constriction of the uterine horn (arrows); a—tumefied ovaries, b—nontumefied ovaries.

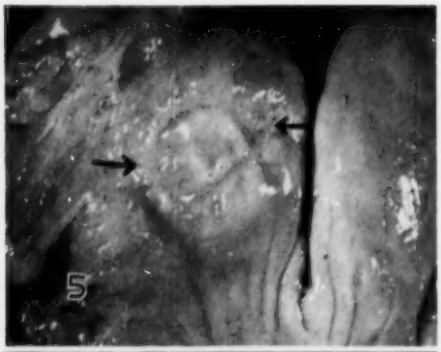
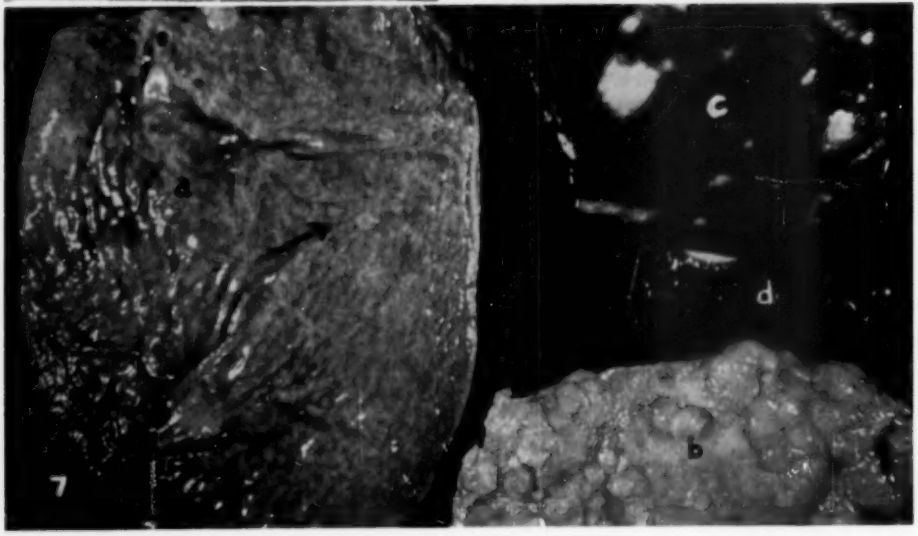


Fig. 8—Adenocarcinoma of the uterus of a 7-year-old cow, case D 1354.

Fig. 5, 6, and 7—Adenocarcinoma of the uterus with metastases of a 12-year-old Hereford, case D 2786. The arrows in figure 5 indicate primary tumor; in figure 6, borders of incised tumor; in figure 7, larger splenic lesions (a—spleen, b—omentum, c—liver, d—incised surface of the liver).



pressed and glossy appearance of the serosa overlying the tumor (arrows, fig. 5). There was no visible extension of the tumor through the surface mucosa of the endometrium (fig. 6, incised lesion with arrows indicating its borders) and the uterine lumen was still patent. Greyish, raised, nodular or smooth-surfaced growths

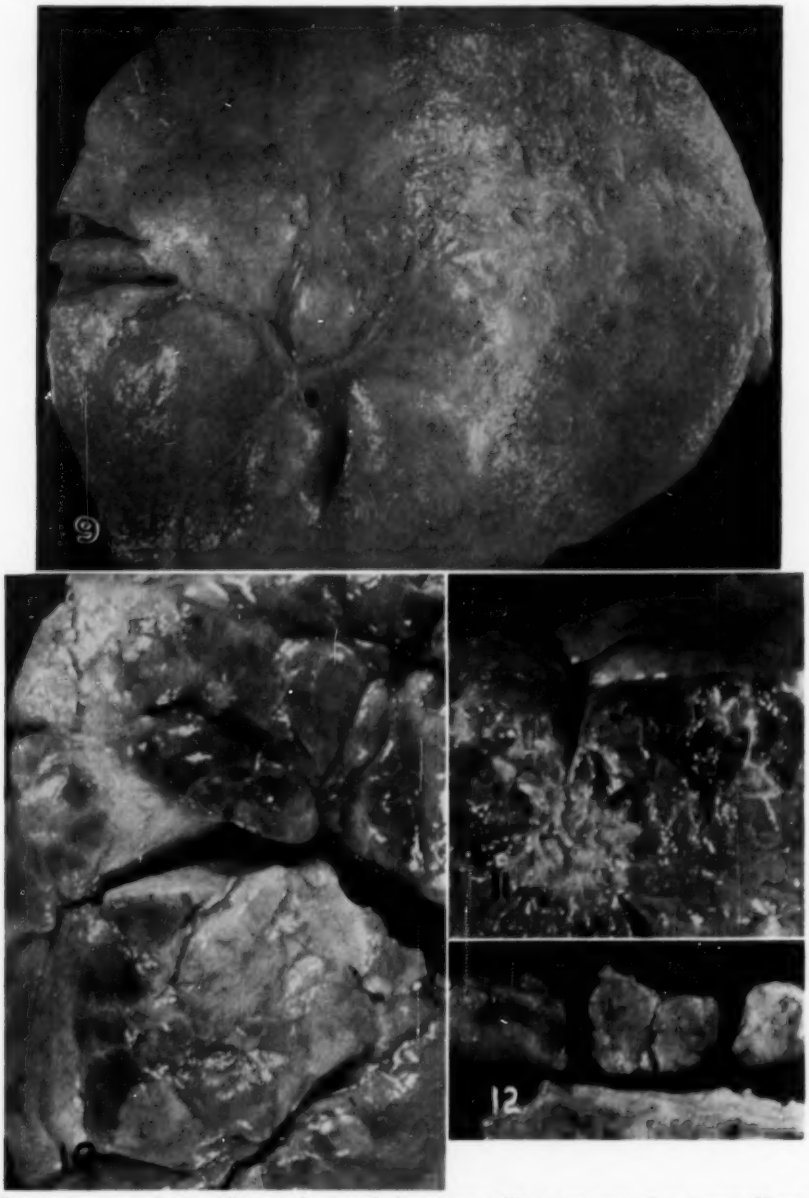


Fig. 9, 10, 11, and 12—Adenocarcinoma of the uterus with metastases of a 7-year-old cow, case D 2973.

(5 to 30 mm. in their greatest diameter) were found on the broad ligament of the uterus and on the serosal surface of the uterus, omentum, peritoneum, liver, spleen, and other abdominal organs (fig. 7, spleen [a], omentum [b], and the serosal surface [c] and incised surface [d] of the liver; larger splenic lesions indicated by arrows). Some of the splenic and liver growths were invading underlying parenchyma. Numerous, solid, yellow, homogenous nodules (5 to 20 mm. in diameter) were present in all lobes of the lungs. There were also large tumor masses in the sublumbar, internal iliac, and deep inguinal lymph nodes.

Necropsy of 7-Year-Old Cow, Case D 2973.—One horn of the uterus was greatly distended with a purulent exudate and a portion of its wall was replaced by a flattened, extremely fibrous, greyish growth (15 by 15 by 2 cm.) which blended into the adjacent portions of the uterus (fig. 9, endometrial surface of lesion). Many grey or yellow, fibrous, irregularly shaped growths were scattered throughout the parenchyma of all lobes of both lungs (most were 5 to 40 mm. in their greatest diameter). Near the pleural surface of the lung, the tumor sometimes formed crater-like, fibrous lesions (fig. 10). An incised lung lesion is shown (fig. 11). Many tiny, shotlike tumors (2 to 5 mm., dia.) were present in the mediastinal pleura in both pleural cavities. Some were thrombosed vessels (fig. 25). The bronchial and anterior and middle mediastinal lymph nodes (fig. 12) were enlarged and fibrous and contained greyish or yellowish tumor masses.

Lung and Lymph Node Metastases of an Adenocarcinoma of the Uterus.—Necropsy of a 10-Year-Old Hereford Female, Case D 2031.—A pale yellow, firm mass (2 by 2 by

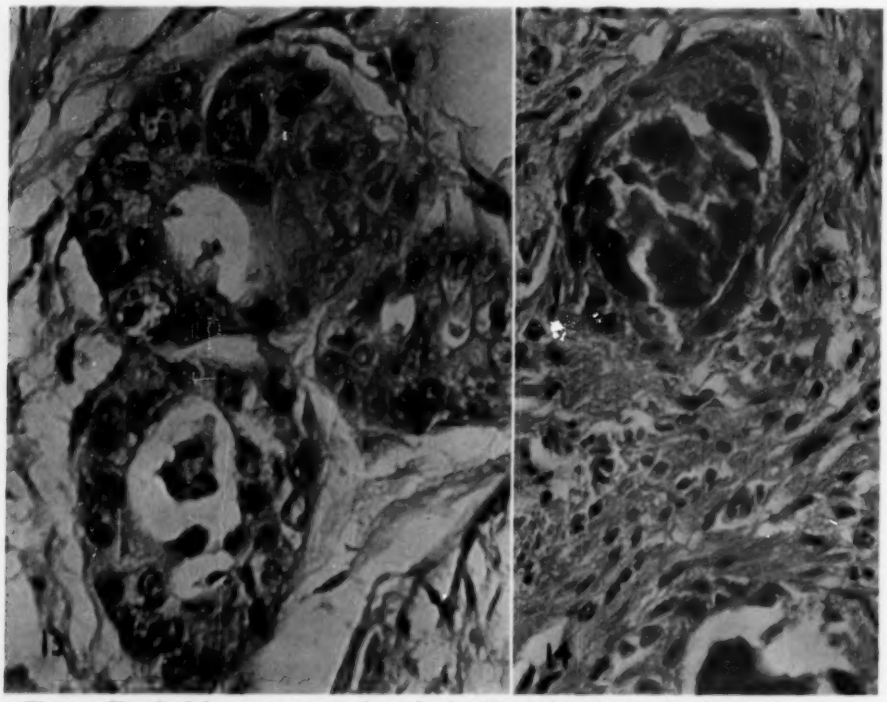


Fig. 13—The glandular arrangement of neoplastic cells consistently seen in adenocarcinoma of the uterus. Note the variation in size and shape of the nuclei and the uneven distribution of chromatin in same. Uterus (bovine case D 2332). Hematoxylin-eosin. x 625.

Fig. 14—Calcium deposited within neoplastic glandular spaces in an adenocarcinoma of the uterus. Small, focal areas of calcification were also found in the connective tissue stroma of this tumor. See figure 9 for a gross picture of this lesion. Uterus (bovine case D 2973). Hematoxylin-eosin. x 500.

1 cm.) was continuous with the walls of both cornua of the uterus about 2 cm. from their bifurcation from the body of the uterus. Yellow, granular, partially calcified nodules were found in the internal iliac lymph node and the bronchial lymph nodes (fig. 24) and as multiple lesions in both lungs (arrows in fig. 23 show a single pulmonary lesion). Note the similarity of the gross appearance of the lung lesion to the two illustrations (fig. 36, 37) of primary lung tumors.

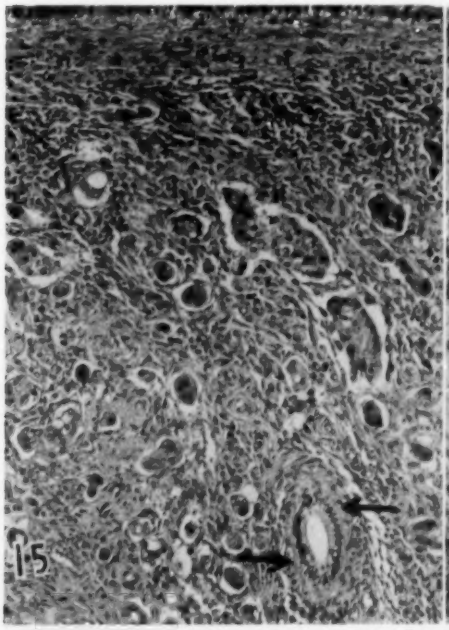
NECROPSY FINDINGS IN SELECTED CASES OF ADENOMA OF THE LUNG

Cut Surface of an Adenoma of the Lung.—Necropsy of a 2-Year-Old Hereford Female, Case D 1241.—Both lungs were studded with soft, greyish, rounded masses (up to 1.5 cm. in diameter). The thoracic lymph nodes appeared normal. The main bronchi and their larger branches were free of tumor (*see* fig. 36).

Necropsy of a 2-Year-Old Hereford Steer, Case D 2155.—Many greyish white, noncalcified, firm masses were scattered throughout all lobes of the lungs. A few of the larger growths were soft and cheese-like and contained small, central cystic spaces. The tumors were not found in the mucosa of the trachea or main bronchi. Most of the growths varied from 1 to 3 cm. in their greatest diameter. Many of the body and visceral lymph nodes were injected and hyperplastic but contained no recognized metastases (*see* fig. 37).

MICROSCOPIC DATA

A significant finding in this study has been the observation that, in the metastases of the bovine uterine adenocarcinomas, there is maintained a degree of differentiation and a limit to anaplastic changes. This does not imply that evidences of malignancy were not easily recognized. Rather, we would advance only the observations that in no case was a glandular



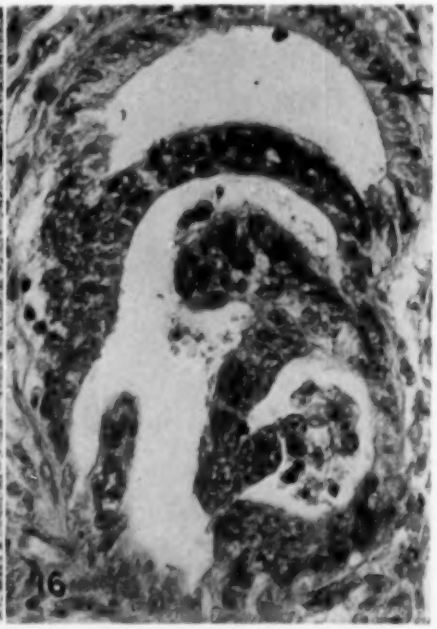


Fig. 15—Adenocarcinoma of the uterus. Groups of tumor cells are infiltrating beneath an intact uterine surface epithelium. Note the hyperplastic uterine gland (arrows) which is still recognizable in the distorted endometrium. Uterus (bovine case D 2691). Hematoxylin-eosin. x 125.

Fig. 16—Is this an early neoplastic transformation or a neoplastic invasion of a gland? Note the intact glandular epithelium (arrows) and a papillary structure (lower left corner). Uterus (bovine case D 1210). Hematoxylin-eosin. x 425.

pattern not seen in the metastases and that solid epithelial groupings of cells in the metastatic growths were minimal. The consistent finding of a glandular pattern was particularly important in view of the tendency for many adenocarcinomas from other organs to form anaplastic masses of neoplastic epithelial cells in a reasonably high percentage of their primary sites and metastases. The undifferentiated-cell and mixed-cell types of primary lung tumor illustrated (fig. 43, 44, 46, 47, 48) and discussed in this article are examples of the lack of differentiation found in other types of bovine tumors. In almost direct contradiction to our findings of a consistent glandular pattern are the observations made by pathologists on cases of adenocarcinoma of the uterus in women (Tudhope and Chisholm's paper [24] is an excellent example). They recognized extreme anaplasia so frequently that they deplored the mistakes made in diagnosing diffusely cellular adenocarcinomas of the uterus as sarcomas.

Varying degrees of glandular differentiation in the primary lesion of an adenocarcinoma of the bovine uterus were seen (fig. 13, 15, 19, and 22). There was neither a consistent size or shape to any of these recognizable glandular structures nor, conversely, a transformation into some other distinctive pattern. Neoplastic glands in which the nuclei of individual cells assumed a basal position (fig. 34) were not frequently encountered. One soon realizes the necessity of studying uterine adenocarcinomas as a group rather than to attempt to visualize their similarities to the normal tubular glands of the endometrium. While cuboidal, neoplastic epithelium was frequently present, a stratified epithelium was not uncommon and could be related to the rapid growth of the tumor. It should be mentioned, however, that stratification is seen in normal surface epithelium and tubules of the uterine glands, as has been pointed out by Hatch.[10] Stratification and rapid growth sometimes obliterated the central cavity of the gland but usually left

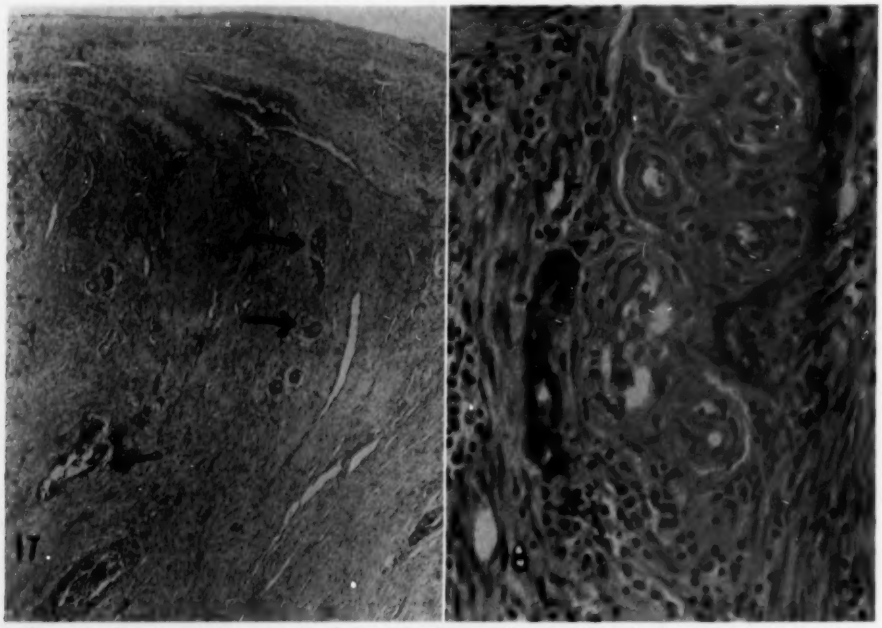


Fig. 17—The lymphatic spread of uterine adenocarcinomas through the muscularis to the serosa is facilitated by abundant lymphatics in these areas. Note the groups of tumor cells plugging thin-walled vessels (arrows). Uterus (bovine case D 2907). Hematoxylin-eosin. x 45.

Fig. 18—Adenocarcinoma of the uterus. Note the groups of tumor cells arranged in tissue spaces near thick-walled blood vessels in the myometrium. Uterus (bovine case D 2786). Hematoxylin-eosin. x 250.

a small but recognizable space (fig. 13). The rapid growth also may contribute to the formation of the cellular, often necrotic, debris found within many of the glands (fig. 22, 29, 34). The epidermoid (squamous) metaplasia seen rather commonly in human cases was not observed in any of the tissues examined in this study. Most of the growths had a very fibrous stroma (fig. 14, 26, 27, 29, 31) and an infiltrate in the tumefied areas of chronic inflammatory cells, including lymphocytes and eosinophils, was not uncommon (fig. 19, 34). Focal calcification (fig. 14) was not usually seen. Occasionally, microscopic fields were found containing tumor giant cells (fig. 35) in which the neoplastic cells appeared to be a reactive cell of a granulomatous process. In our cases, there was little evidence of progressive growth through and on the surface epithelium of the uterus. The impression from the present study was that the tumor usually was centered deep in the endometrium and spread laterally under an intact surface epithelium (fig. 15). In this manner, many non-neoplastic uterine glands (fig. 15, 16) might have been incorporated into the growths and the distorted appearance of a few may actually have falsely suggested that they had undergone early neoplastic change. While a generalized hyperplasia of residual uterine glands in some of these involved areas was seen, the atypical or adenomatous hyperplasia (carcinoma *in situ*) described by Speert [2,29] and Hertig, Sommers, and Bengloff [12] in human adenocarcinoma of the uterus has never been encountered in our limited studies of sup-

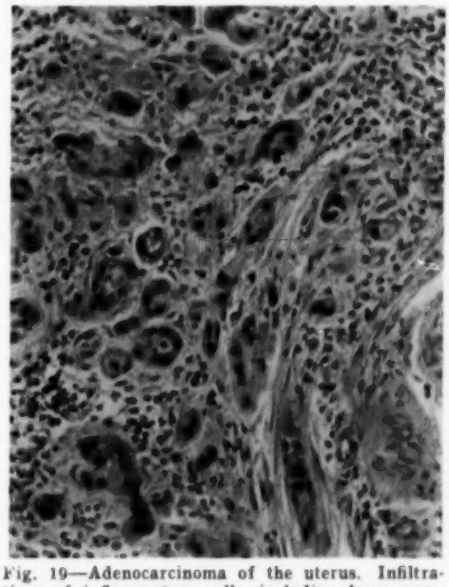


Fig. 19—Adenocarcinoma of the uterus. Infiltrations of inflammatory cells, including large numbers of lymphocytes and eosinophils, were often prominent in the uterine lesions. Uterus (bovine case D 2786). Hematoxylin-eosin. x 200.

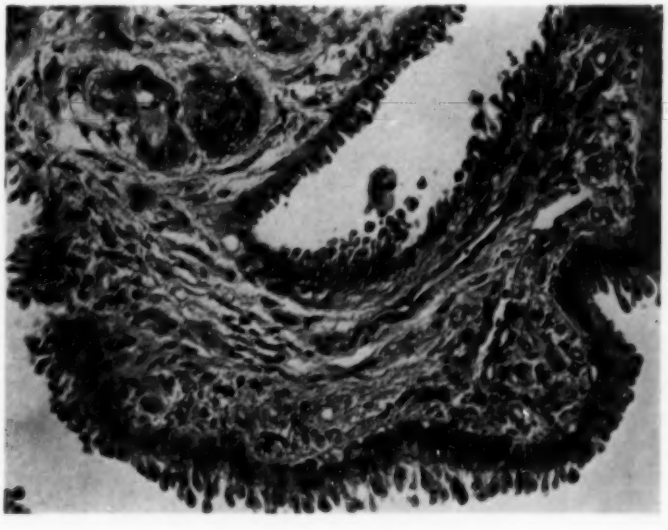


Fig. 20—Metastasis of an adenocarcinoma of the uterus. Note the nests of tumor cells in the submucosal tissues of a fallopian tube suggesting progressive spread from uterine lesions by lymphatics. Fallopian tube (bovine case D 2477). Hematoxylin-eosin. x 260.

posedly normal and diseased bovine uteri. These changes in the uterus in women are represented by distinct and recognizable zones in the endometrium. The involved glands are enlarged and irregularly shaped. In addition, the cells lining the glands are very large with paler than normal and eosinophilic cytoplasm.

The growing uterine tumor in the cow apparently at some stage in its development is able to spread through the muscular layers of the myometrium (fig. 17 and 18). In most of our cases where the tumor has broken through into the myometrium, groups of tumor cells were found at the perimeter of the thick-walled blood vessels which form many vascular channels through the myometrium. Sometimes, these tumor cells appeared to be in thin-walled vessels comparable to lymphatics or dilated veins. They were also seen as clumps of cells near the adventitia of the vessels and it was problematical whether they were confined by smaller and more difficult to demonstrate vessels or were infiltrating through the supporting tissues of these vascular channels. In figure 17, the neoplastic cells are close to the serosa. At the borders of the primary lesions, the myometrium beneath intact endometrium was frequently focally infiltrated by the tumor (fig. 22). The appearance of our few examples of metastasis to the fallopian tubes (fig. 20) was also suggestive of lymphatic or vascular spread. Willis[37] and other pathologists believe that, in many of their human cases, fragments of uterine carcinomas pass through the lumen of the fallopian tubes and enter the peritoneal cavity. Such a route of metastasis is impossible to disprove in most of our 14 bovine cases with peritoneal lesions. However, the lack of or only limited growth of the bovine tumors on the surface of the endometrium suggested that transtubal metastasis might be of only minor importance in the cow. It is of interest that Novac[20] and many other investigators of the pathogenesis of human adenocarcinoma of the uterus had early postulated, and still receive support for their opinion, that the lymphatics are the most important route of metastasis to the fallopian tubes, ovary, and peritoneum. A study of early bovine cases should establish whether the tumor is originally superficial, as in most human cases, or whether it can arise deep in the endometrium, as we suspect from a review of our material from advanced cases.

The marked annular constriction (fig. 1, 3, 5), seen in many of our primary lesions of uterine carcinoma and which microscopically showed neoplastic invasion to near a serosal surface (fig. 17), suggested that a tumor might break through that limiting membrane and establish peritoneal implants. However, the lack of implants and serosal fibrosis in the constricted areas (although the underlying lesion in the muscularis may be very fibrotic) and the observa-

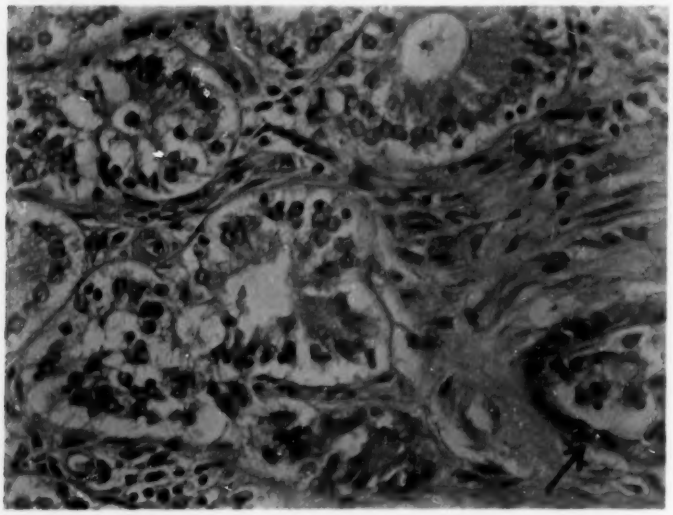


Fig. 21—Neoplastic cells of a uterine adenocarcinoma sometimes infiltrate through the muscularis and are found at the base of the glands of the endometrium. Note the neoplastic gland (arrow) near morphologically normal glands. Uterus (bovine case D 2824). Hematoxylin-eosin. x 350.

tion that some of our most constricted lesions had no peritoneal metastases indicated that there had not been a rupture of the uterine wall in any of the lesions available for detailed examination. The enlargement, fibrosis, and possibility of rupture and liberation of tumefied tissues from lymph nodes has already been discussed. An example of the extreme fibrosis often associated with these lesions is shown (fig. 27).

Metastatic lung lesions of an adenocarcinoma of the uterus seem to differ from their primary lesion and other metastases only in their pattern of growth in this different parenchyma. Tumor cells in a peribronchial lymphatic are shown (fig. 28) only a short distance from the border of a larger metastatic lesion. Monlux [15] in his paper "Primary Pulmonary Neoplasms in Domesticated Animals" suggested that not only tumors but bacterial and virus diseases spread most commonly through the peribronchial tissues and that these tissues seem to be the path of least resistance for such extensions. Metastases of a uter-

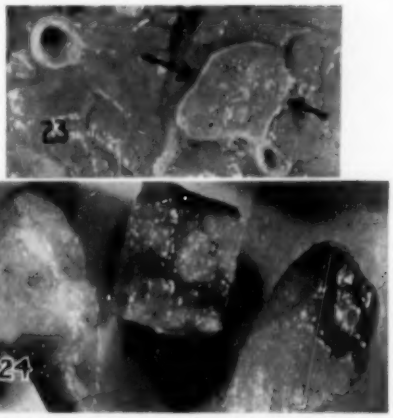


Fig. 23 and 24—Lung and lymph node metastases of an adenocarcinoma of the uterus of a 10-year-old Hereford, case D 2031. Arrows in figure 23 show a single pulmonary lesion.

Fig. 22—Lower power of figure 21 showing the diffuse invasion of the myometrium beneath normal uterine glands. The neoplastic gland included in figure 21 is indicated by arrows. Uterus (bovine case D 2824). Hematoxylin-eosin. x 50.

Fig. 25—A tumor thrombus in a large thin-walled vessel in the mediastinal pleura of a cow. The point of attachment of the thrombus is not shown in the photomicrograph. Mediastinum (case D 2973). Hematoxylin-eosin. x 45.

TABLE 5—Primary Bovine Epithelial Tumors of the Lung

Case No.	Estimated age (yr.)	Sex	Breed (when known)	Distribution of pulmonary lesions	Location of lymph node metastases	Diagnosis
D 2795	10	F	.....	Multiple-focal	Mediastinal	Undifferentiated-cell carcinoma
D 2761	old	F	.....	Multiple-focal	Bronchial, mediastinal	Undifferentiated-cell carcinoma
D 2558	9	F	Hereford	Multiple-focal	Bronchial, mediastinal, cervical, retropharyngeal	Undifferentiated-cell carcinoma
D 2411	5	F	.....	Multiple-focal	Bronchial, mediastinal, cervical	Mixed-cell carcinoma
D 2155	2	S*	Hereford	Multiple-focal	—	Columnar-cell adenoma
D 1666	2	F	.....	Multiple-focal	—	Columnar-cell adenoma
D 1241	2	F	Hereford	Multiple-focal	—	Columnar-cell adenoma
D 977	8	F	.....	Primary-focal	Bronchial, mediastinal	Mixed-cell carcinoma
D 389	2	F	.....	Multiple-focal	—	Columnar-cell carcinoma

* Steer; F female; — no recognized metastasis.

ine tumor are shown (fig. 30 and 32) in the tissues adjacent to bronchioles. As might be expected, some of the metastatic lung lesions were well encapsulated (fig. 31), most had a very noticeable fibrous stroma (fig. 29, 34, 35), and a few of the more rapidly growing metastases were still invading adjacent tissues (fig. 33). The fibrotic changes in the primary growth and the metastases were so consistent that Ottosen [22] would seem to have been quite justified in designating the tumor in both the uterus and lung as scirrhous adenocarcinoma.

Our series of nine primary bovine epithelial tumors of the lung (table 5) is admittedly small but does demonstrate several distinct types of growths which are easily differentiated from lung metastases of uterine carcinomas. It is from this

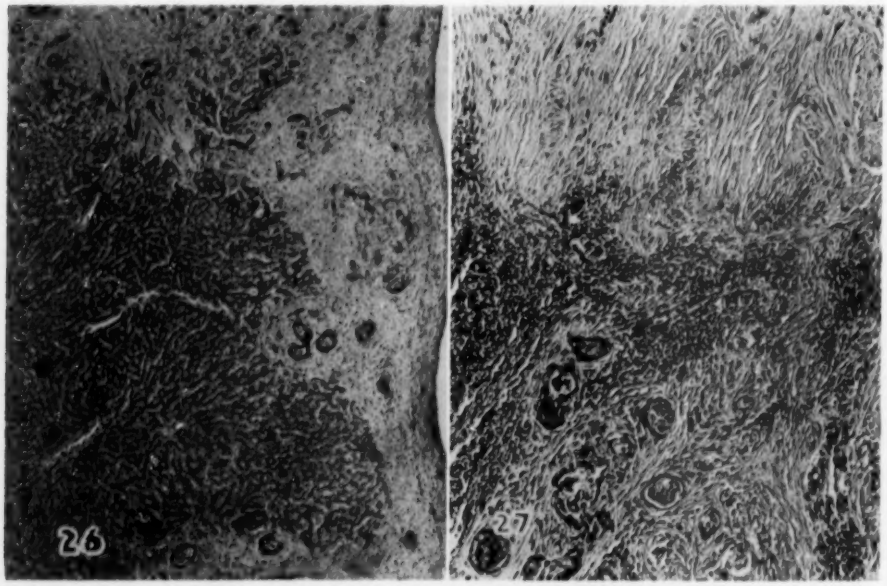


Fig. 26—Metastasis of an adenocarcinoma of the uterus. The progressive invasion of liver parenchyma was probably the result of growth of tumor implant. Note the glandular arrangement of the tumor cells and the prominent cirrhosis. The gross appearance of the liver is shown in figure 7. Liver (bovine case D 2786). Hematoxylin-eosin. x 45.

Fig. 27—Metastasis of an adenocarcinoma of the uterus. This area in a tumefied lymph node shows the fibrosis often associated with metastases. Note the thick bands of collagen. Lymph node (bovine case D 2973). Hematoxylin-eosin. x 50.

standpoint that their presentation is considered worthwhile. The nine cases do not include several probable primary lung tumors in which incomplete necropsies or marked anaplastic changes caused reasonable doubt as to their origin. One of us (W.S.M.) has reviewed and discussed 32 reported cases of bovine lung tumors and compared them with other lung tumors in domesticated animals and in man. Monlux, in that article,[1] suggested the division of pulmonary carcinomas into three groups on the basis of distribution of lesions: (1) primary-focal, (2) multiple-focal, and (3) diffuse-infiltrating. Each of these groups was then subdivided into four types: (1) squamous-cell, (2) columnar-cell, (3) mixed-cell, and (4) undifferentiated-cell. The adoption of this classification rather than the classification advocated by Ewing† in his widely quoted text[5] is supported by present day observations and research. Lung epithelium and bronchial gland epithelium have been shown to change under various pathological conditions from one cell type to another. In addition, and probably more convincing to the pathologist, there is a pleomorphism exhibited by even solitary neoplastic pulmonary growths in which glands or different types of epithelial structures or both exist side by side. To explain the inclusion of these variable components, one must

† Ewing thought most epithelial lung tumors could be identified in respect to their origin on the basis of their morphology. His classification associated individual tumors with a specific cell of origin in either mucous glands of the bronchi, cells lining the bronchi, or the epithelium which lines the pulmonary alveoli.

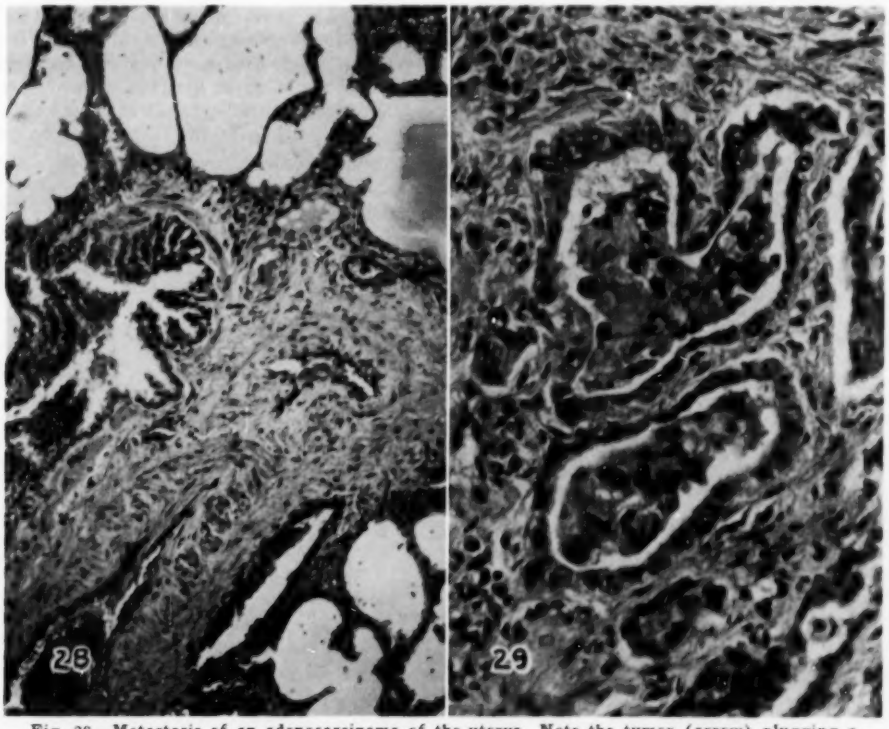


Fig. 28—Metastasis of an adenocarcinoma of the uterus. Note the tumor (arrow) plugging a thin-walled vessel near a bronchiole. Lung (bovine case D 2973). Hematoxylin-eosin. x 85.
Fig. 29—Lung metastasis of an adenocarcinoma of the uterus. The central spaces of neoplastic glandlike structures contain partially necrotic cellular debris. Lung (bovine case D 2973). Hematoxylin-eosin. x 165.

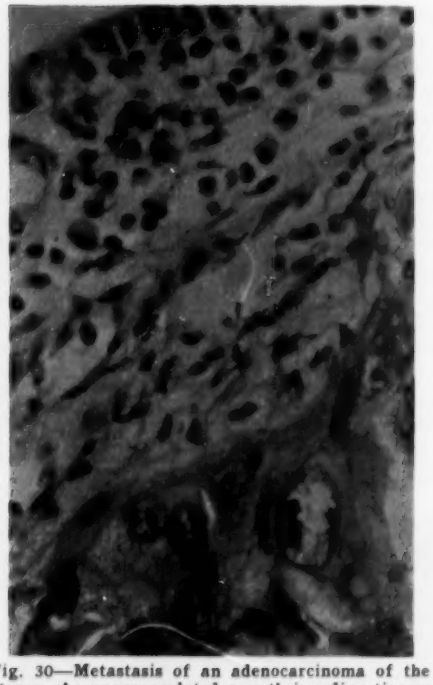


Fig. 30—Metastasis of an adenocarcinoma of the uterus. An unencapsulated growth invading tissues adjacent to a large bronchiole. Lung (bovine case D 2031). Hematoxylin-eosin. x 266.

either acknowledge multiple cells of origin for the growth or assume that there are neoplastic transformations of a single cell of origin. Finally, the origin of epithelial lung components from similar embryological cells seems to justify the abandonment of the older terminology except in instances where the names have descriptive value.

In our cases of primary lung tumors (table 5), we had eight of the multiple-focal group and one of the primary-focal group. In the group of bovine lung tumors reviewed by Monlux, only 26 of the 32 were sufficiently described to be given a group designation: 14, or over half, were of the multiple-focal group, 11 of the primary-focal group, and one of the diffuse-infiltrating group. These statistics would seem to indicate that some primary-focal lung tumors were being overlooked in our survey. While this may be true, it was of interest that seven of the 11 cases in the primary-focal group in the Monlux [18] review had been reported before the year 1912, and six of these by Trotter who diagnosed round cell carcinoma frequently and, in describing them, gave excellent descriptions of malignant lymphomas. Among the recent investigators, Sjolte [27] reported a total of seven primary lung tu-

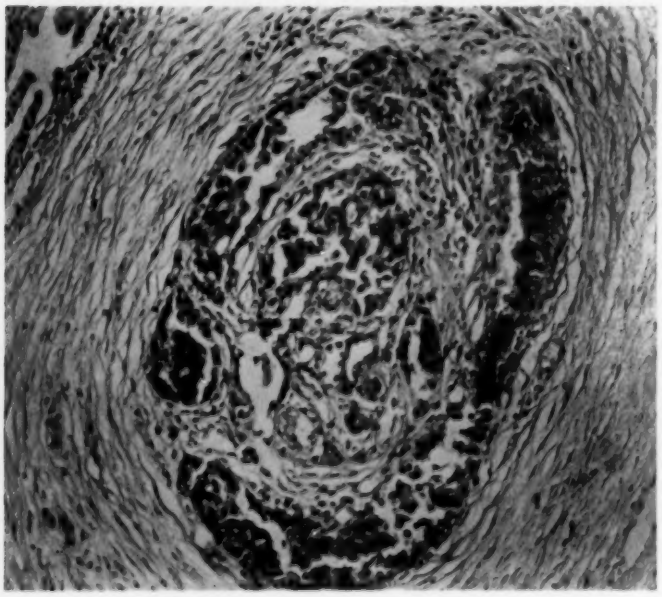


Fig. 31—Metastasis of an adenocarcinoma of the uterus. A thickly encapsulated lung lesion. A gross picture of a larger, encapsulated lung metastasis from this same animal is shown in figure 23. Lung (bovine case D 2031). Hematoxylin-eosin. x 85.

mors and all were of the multiple-focal group. Buer [2] listed five primary lung tumors with two being multiple-focal and three being primary-focal. The bronchogenic carcinoma of the epidermoid type reported by Savage and Isa [24] in 1953 was not considered by us to be a primary lung tumor. The resemblance of the lesions described to metastases of a carcinoma of the eye or its appendages, the metastatic character of the growths, and the examination of only thoracic viscera by the authors were the basis of our doubt in their conclusions. Plummer's recent description [23] of two cases diagnosed as primary bovine lung tumors was brief, not illustrated, and difficult to evaluate on the basis of data presented. In our nine cases, four occurred in young animals and none of these had extrapulmonary metastases. Their appearance and the multiple-focal distribution of lesions suggested a possible hamartial origin for each tumor.

Three of these four lung tumors, which were of the columnar-cell type, would be excellent examples of a tumor of peribronchial glands under the Ewing terminology. The 3 animals, 2 heifers and 1 steer, were in apparent good condition and 2 years of age at the time of slaughter. The lesions were found in all lobes of the lungs and were remarkably similar in gross appearance (fig. 36, 37). When sections were made of the multiple tumors, the microscopic pattern of growth was almost identical and quite distinctive (fig. 38, 39, 40, 41). A markedly papillary type of growth was found in all of the larger lesions (fig. 38, 40) with the epithelium varying from a columnar cell to a distinctly stratified variety (fig. 39). Instead of evidence of metastatic spread of the tumor, small, nonencapsulated growths (fig. 41) were seen distinctly separated from the larger tumors. Only a cuboidal or low columnar epithelium was seen in these smaller growths and this epithelium suggested an earlier and more benign stage of growth rather than metastases. Their appearance helped to confirm our suspicions that the

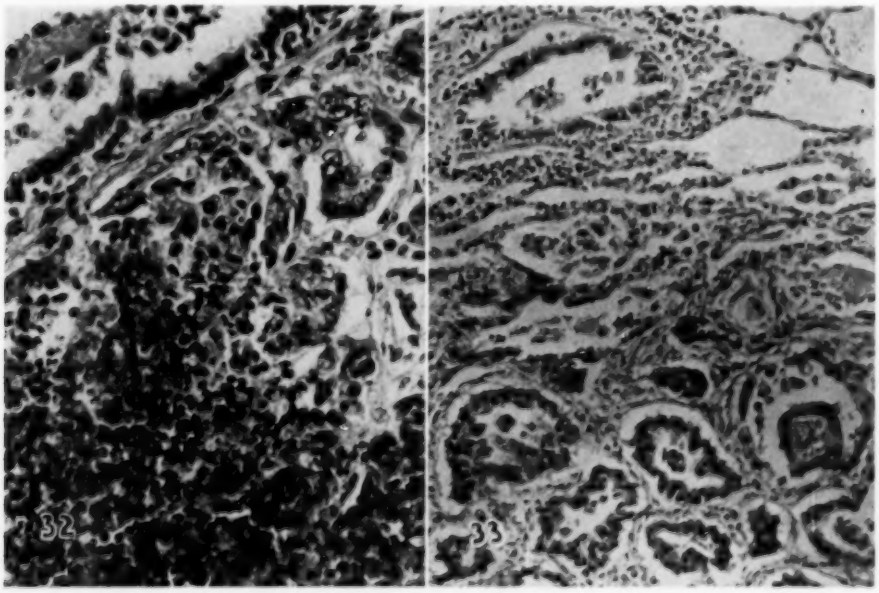


Fig. 32—Metastasis of an adenocarcinoma of the uterus. Necrotic changes are not limited to debris within glands in many of our cases. Sometimes, large areas of the tumors become necrotic. Note the necrotic area in this growth adjacent to the persisting bronchiole. Lung (bovine case D 2691). Hematoxylin-eosin. x 165.

Fig. 33—Metastaris of an adenocarcinoma of the uterus. Lung parenchyma including a bronchiole can be seen adjacent to the unencapsulated border of the tumor. Lung (bovine case D 2031). Hematoxylin-eosin. x 85.

growths in each lung were multicentric in origin and possibly a result of a neoplastic change in a developmental defect (a hamartoma). The lack of evidence of malignancy, as already stated, and the finding of three cases without metastases were the basis for our present designation as adenomas.

Our fourth case in a young animal was a 2-year-old female which had multiple lesions in all lobes of the lungs. There was a remarkable degree of consistency in the microscopic appearance of these lesions (fig. 45). The tumor cells were of a uniform columnar-cell type.

In cow D 977, a primary tumor of the lung and its satellite growths were confined to a single apical lobe (fig. 42, 43, 44). This animal was an 8-year-old female and the tumor was our only example of a carcinoma of the primary-focal group. In both the lung and in its bronchial and mediastinal lymph node metastases, the columnar-cell and undifferentiated-cell components of this mixed-cell tumor were recognized. The undifferentiated-cell type of lung tumor probably varies more in appearance than other types of lung tumors (fig. 46, 47, and 48 illustrate two distinct patterns of growth encountered in this study). We had no tumors of the squamous-cell type in our series (table 5) and it is of interest that Buer [2] reported all of his five cases to be of a columnar-cell type. Sjolte [27] described two squamous-cell, one columnar-cell, and four undifferentiated-cell type of tumors in his report of seven cases.

DISCUSSION

It is unfortunate that clinical observations could not have been made on the animals afflicted with uterine carcinoma in our series. In the available reports of bovine adenocarcinoma of the uterus published during the last 30 years, we could find records of clinical examinations on 1 animal with a possible uterine carcinoma observed by Udall, Fincher, and Cushing,[35] 1 reported by Ball and Boudet,[1] and 4 included in Ottosen's series [21, 22] of 15 cases.

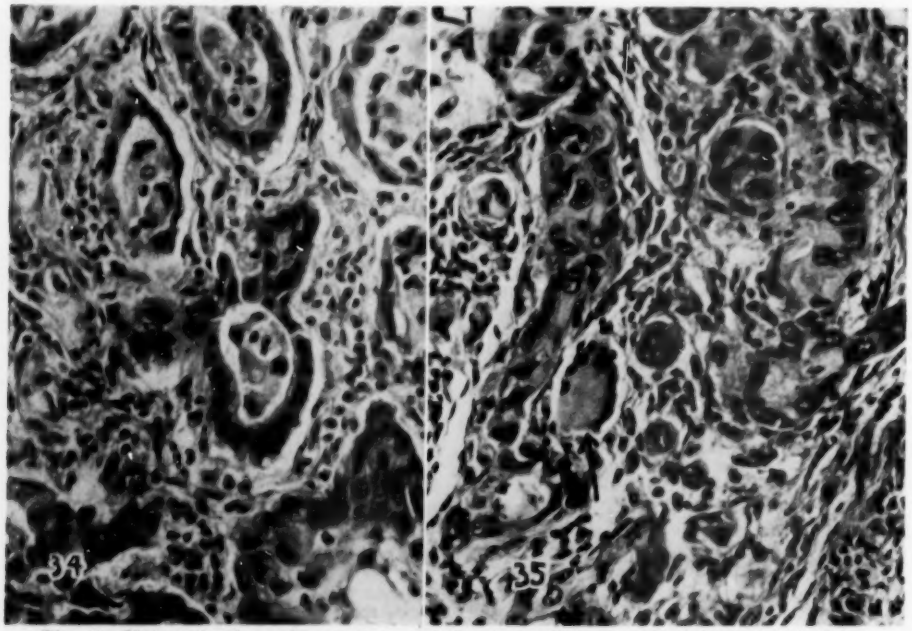


Fig. 34—Metastasis of an adenocarcinoma of the uterus. Note the cuboidal tumor cells lining neoplastic glandular structures and the tendency of their nuclei to assume a basal position. Lung (bovine case D 2662). Hematoxylin-eosin. x 165.

Fig. 35—Metastasis of an adenocarcinoma of the uterus. The giant cell (arrow) and the chronic inflammatory cells suggest a granulomatous reaction in which the tumor was actively participating. Lung (bovine case D 2662). Hematoxylin-eosin. x 165.

The meager information on these animals would suggest that sterility was not always associated with the disease.[1] In other instances,[21,22] sterility was recognized only a few months before it became obvious that the animal was in the terminal stages of a fatal neoplastic illness. We have made limited examinations of nontumefied ovaries and the intact portions of the endometrium in available cases and have recognized no significant anatomical deviations from normal cyclical changes.

In many well-documented cases of adenocarcinoma of the uterus in women, there is a coincidental or direct relation of the disease to endocrine imbalance and particularly to excessive estrogen stimulation. Among the findings listed in reports[11,12,28,29] as present concurrently with or preceding adenocarcinoma of the human uterus were endometrial lesions producing symptoms of disease varying from endometrial hyperplasia to carcinoma *in situ* and large, smooth-surfaced ovaries with cortical fibrosis and subcortical follicular cysts. A history of previous estrogen administration, particularly to patients in the menopause, and a nulliparous sexual background was noted also in many of these human cases. Perhaps it is as well that similar clinical phenomena do not develop frequently in the cow since, in some medical circles, such findings in human cases are regarded to be as misleading as they are helpful until a final diagnosis is made. These investigators point out that there may be evidence of estrogen imbalance in many non-neoplastic conditions. We have found no associated granulosa-cell or other ovarian tumors in our series of bovine uterine carcinomas and find none reported in the literature. This association is present in some human cases and again is suggested in recent publications to be related to endocrine imbalance. In the cases of bovine granulosa-cell tumor of the ovary examined at the Denver laboratory, an appreciable endometrial hyperplasia was seen in less than half of the uteri. Few cysts were present in the hyperplastic glands in the endometrial lesions and a swiss-cheese pattern was not observed. McEntee[17] in his studies on bovine reproductive diseases has observed endometrial hyperplasia only in some cows with cystic ovaries and granulosa-cell tumors. He also

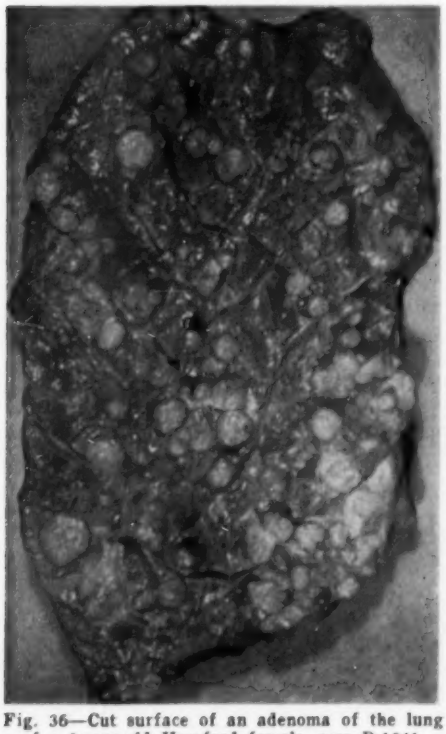


Fig. 36—Cut surface of an adenoma of the lung of a 2-year-old Hereford female, case D 1241.

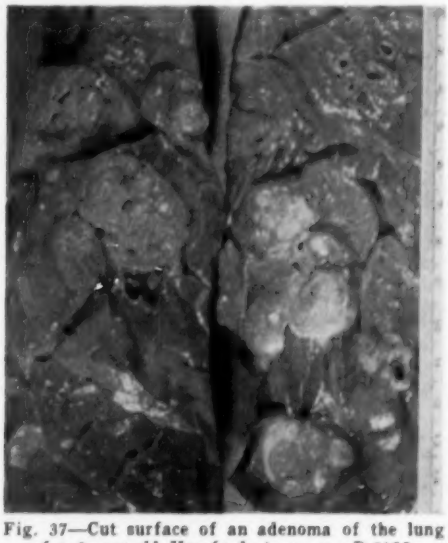


Fig. 37—Cut surface of an adenoma of the lung of a 2-year-old Hereford steer, case D 2155.

reports that the hyperplasia was not as pronounced as that which often occurs in human and canine cases.

Fig. 39—Adenoma of the lung. Higher power of a papillary-type growth similar to the one shown in figure 38. This field was chosen to show the stratified character of portions of the epithelium. The gross appearance of this tumor is shown in figure 37. Lung (bovine case D 2155). Hematoxylin-eosin. x 266.

It is doubtful if any observations in cattle except advancing age, sterility, physical disability, illness, or discovery of suspicious lesions or exudates on physical examination could be helpful in leading to a diagnosis or be correlated with a diagnosis of adenocarcinoma of the uterus at necropsy. Many of the present diagnostic methods employed in detecting early human carcinomas of the uterus would seem to have only a limited application in bovine cases.

Even the most accurate statistics[39] on the occurrence of fatal cases of uterine tumors in women can be somewhat misleading. Besides errors in diagnosis and reporting, there is also an approximate halving in numbers of all tumors of the genital tract unless the statistics of each sex are reported separately. Willis[47] points out that carcinomas of the uterus, stomach, and breast are believed to be the most common cause of death from cancer in women. Cattle husbandry practices result in a sex predominance of females when the animals are older and in what might be called "the cancer age." Finally, if we accept that there is ample precedence in human statistics to say that a high incidence of uterine tumors in cattle would not be unusual from a comparative standpoint, we should mention the relative infrequent occurrence

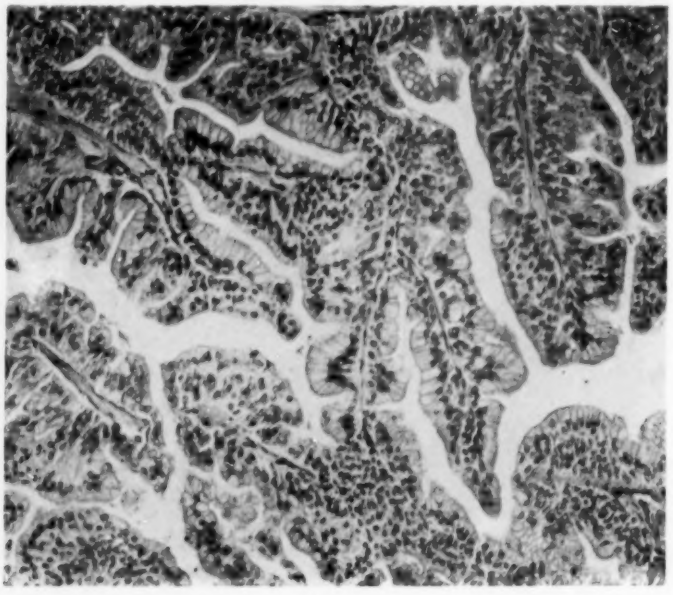


Fig. 38—Adenoma of the lung (columnar-cell type). Note the columnar or stratified appearance of the epithelium and the frondlike papillary manner of growth of this neoplasm. A gross picture of this tumor is shown in figure 36. Lung (bovine case D 1241). Hematoxylin-eosin. x 150.

Fig. 40—Adenoma of the lung. An encapsulated border of a tumor similar in appearance to the growth shown in figure 38. Lung (bovine case D 2155). Hematoxylin-eosin. x 50.

Fig. 41—Adenoma of the lung. A small nonencapsulated tumor characterized by a low columnar-cell epithelium. Many similar growths were found in the parenchyma of the lung between larger tumors. The appearance of the larger tumors is illustrated in figures 38, 39, and 40. Lung (bovine case D 2155). Hematoxylin-eosin. x 85.

Fig. 42—Adenocarcinoma of the lung. A field of a mixed-cell type of lung tumor in which columnar-cell lined spaces predominate. Lung (bovine case D 977). Hematoxylin-eosin. x 85.

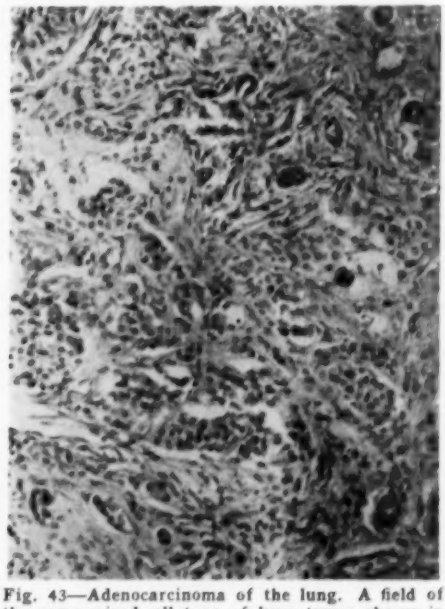


Fig. 43—Adenocarcinoma of the lung. A field of the same mixed-cell type of lung tumor shown in figure 42 in which the undifferentiated-cell type predominates. Lung (bovine case D 977). Hematoxylin-eosin. x 85.

of epidermoid and adenocarcinoma of the cervix in cattle. In women, they represent at least half of the tumors usually statistically listed as uterine carcinomas. Table 2 lists single cases of squamous cell carcinoma and adenocarcinoma of the cervix which are the only cervical carcinomas found at this laboratory. Because of their rarity we have not discussed them except to briefly point out their similarities to uterine carcinomas. A metastatic lesion in the lung of a squamous cell carcinoma of the cervix (fig. 49) had a pattern of metastasis which mimicked our cases of adenocarcinoma of the uterus.

While adrenal and Schwann's cell tumors have a considerably higher occurrence than uterine adenocarcinomas in the cow, there is no comparison in regard to economic loss between the three tumors. Metastases in these first two tumors rarely occur; when they are found in slaughtered animals, there are usually no important secondary lesions of disease unless the growths have reached an extremely large size and produced extensive distortion of adjacent tissues. Subsequent investigations may bear out our contention that the adenocarcinomas of the uterus rank only behind the squamous cell carcinomas of the eye and its appendages and the lymphoid tumors in causing economic loss to the cattle industry. Carcinoma of the uterus should be taken out of the large group of bovine neoplasms regarded by so many as academic oddities because of their benign behavior or infrequent occurrence. This tumor will be detected more often in routine examinations when there is an awareness that it represents an important neoplastic disease of the cow. Important advancements have been made in the detection and knowledge of the behavior of lymphoid tumors and carcinomas of the eye and its appendages in cattle. It is well established that successful treatment of the eye tumors can often be instituted if they are detected in the early stages of their

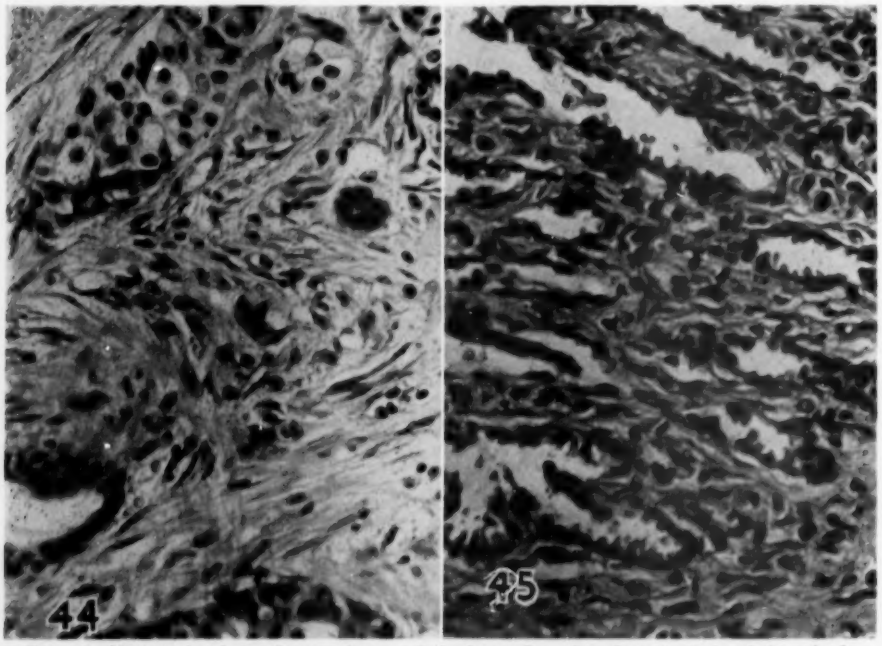


Fig. 44—Metastasis of an adenocarcinoma of the lung. Lymph node metastasis of the mixed-cell type of lung tumor shown in figures 42 and 43, in which both the columnar-cell type and undifferentiated-cell type are seen. Mediastinal lymph node (bovine case D 977). Hematoxylin-eosin. x 165.

Fig. 45—Adenocarcinoma of the lung. A columnar-cell type of lung tumor. Lung (bovine case D 389). Hematoxylin-eosin. x 165.

growth. However, uterine carcinomas would probably warrant almost as guarded a prognosis as is now given for lymphoid tumors. Some of the small primary uterine lesions have had extensive metastases. The resulting value of a sterile and usually old animal would be a deterrent to any radical surgical procedure.

In recent years, European investigators[2, 9, 21, 22, 27] began the study of primary lung tumors and other metastatic adenocarcinomas of the lung in cattle because these lesions were being confused with tuberculosis. They reported the predominance of a large group of metastatic pulmonary carcinomas which were similar in appearance to known uterine tumors and were usually grouped together as scirrhous adenocarcinomas. European pathologists soon suspected that most of these tumors actually had their primary site in the uterus. Our conviction regarding the origin of similar metastatic adenocarcinomas is apparently in complete agreement with these European observations.

An attempt has been made in this article to show how primary epithelial tumors of the bovine lung differ from pulmonary metastases of bovine uterine carcinomas. We chose to limit our differential illustrations to the group of primary lung tumors and to emphasize how the primary site or an indication of the primary site of a metastatic carcinoma is usually evident in the findings of a complete necropsy. The gross appearance of an individual metastatic lesion of some of the other bovine carcinomas which metastasize to the lungs, lymph nodes, or peritoneum (particularly those lesions which are quite fibrotic) can be

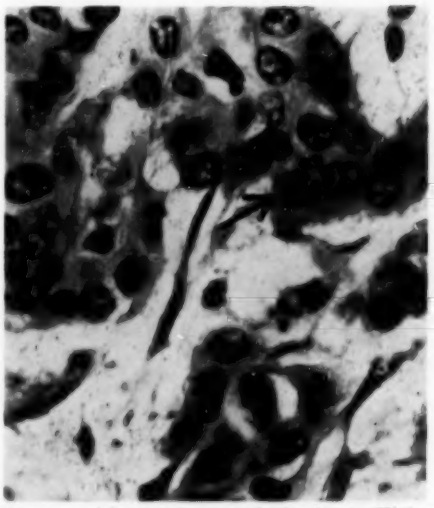


Fig. 47 — Adenocarcinoma of the lung. Higher power of another field of the same section of lung shown in figure 46. Note the mitotic figure (arrow) and the uneven distribution of chromatin in many of the nuclei. Lung (bovine case D 2761). Hematoxylin-eosin. x 500.

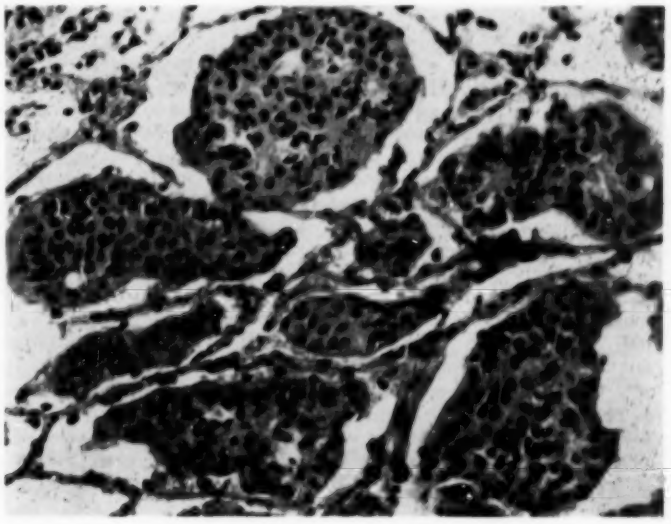


Fig. 46—Adenocarcinoma of the lung (undifferentiated-cell type). Groups of tumor cells are supported by remnants of the alveolar walls. Lung (bovine case D 2761). Hematoxylin-eosin. x 165.

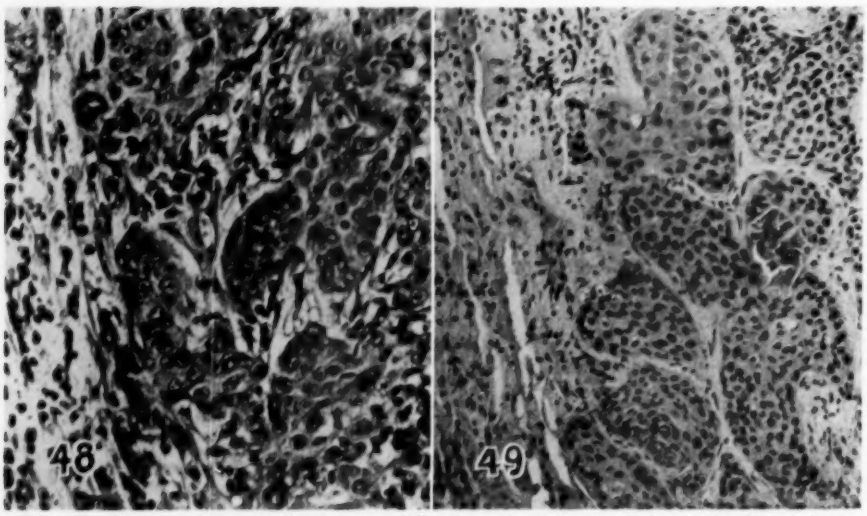


Fig. 48—Adenocarcinoma of the lung (undifferentiated-cell type). This anaplastic neoplasm had metastasized to the bronchial, mediastinal, axillary, retropharyngeal, and the cervical chain of lymph nodes. Lung (bovine case D 2558). Hematoxylin-eosin. x 165.

Fig. 49—Lung metastasis of a squamous cell carcinoma of the cervix. Note the small cornified centers (epithelial pearls). Lung (bovine case D 1743). Hematoxylin-eosin. x 85.

confused with metastases of a bovine uterine carcinoma by even the most experienced investigator. Too often the examination of these metastatic lesions suggest only possible anatomic sites of origin. As an example, we see cases of squamous cell carcinoma of the eye or its appendages with pulmonary metastases. These metastases in the lung are often similar in their gross appearance to pulmonary lesions of metastatic uterine carcinomas. Fortunately, the microscopic appearance of these eye tumors differentiate them from metastases of an adenocarcinoma of the uterus. Like other metastatic carcinomas, reasonably complete necropsy findings in the metastatic squamous cell carcinoma of the eye are helpful in differential diagnosis. The eye lesions (unless previously removed surgically), the massive parotid lymph node metastases, and the enlargement and tumefaction of the mandibular, pharyngeal, atlantal, and cervical lymph nodes can usually be found if there is a pulmonary metastasis. In addition, the metastases to the abdominal lymph nodes and organs are usually minimal or absent.

Dobberstein's observation [4] in regard to the greater frequency of uterine carcinomas in cattle and rabbits than in other domesticated animals, is an indication of the interest that is beginning to be shown in the recognition of the bovine cases. Contributing factors such as breed and environment have not been associated with a higher occurrence of bovine uterine carcinomas. Reports suggest that such factors do contribute to a higher incidence of eye, skin, liver, and fore-stomach tumors in cattle in regional locales in the world. As the references quoted in this paper indicate, investigators from many nations, and undoubtedly dealing with a variety of breeds of cattle, have reported uterine carcinomas. Our finding of uterine carcinomas in Herefords in all but one of the cases where breed was recorded is of interest but does not prove a higher incidence in this breed. In Denver, the majority of the cows slaughtered are of the Hereford breed.

SUMMARY

A study was made of 26 cases of adenocarcinoma of the uterus in cows in which the diagnosis was confirmed by gross and microscopic examination of tissues.

Twenty-two of the uterine carcinomas had recorded lung metastases, 21 had lymph node metastases, and 14 had peritoneal metastases. A consistent gland-like

pattern of growth and secondary sclerotic changes appeared to be very characteristic microscopic features of this tumor.

Sixty-two cases of metastatic adenocarcinomas in cows, represented by at least lung metastases and regarded as probable uterine carcinomas, were compared by the authors with uterine carcinomas. The available metastatic lung, peritoneal, and lymph node lesions in these 62 cases were indistinguishable from metastatic lesions of known uterine carcinomas. The presence of a primary tumor of the uterus could not be established definitely for any of these metastatic carcinomas. The case records of most of the 62 cows were incomplete because either a critical examination of the uterus had not been made or unsatisfactory tissue specimens had been forwarded for laboratory examination. Future investigations of the primary sites of similar metastatic carcinomas should prove the uterine carcinoma to be one of the most common of the metastatic bovine carcinomas.

Since metastatic bovine carcinomas are apt to be confused with primary carcinomas of the lung, the difference in appearance and behavior of nine primary bovine lung tumors was also discussed and illustrated.

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Experimental *Microsporum Canis* Infections in Kittens

GERBERT REBELL; H. F. TIMMONS, M.S.; JOHN H. LAMB, M.D.;
PAUL K. HICKS, B.S.; FRED GROVES, B.S.; ROBERT E. COALSON, Ph.D.

Oklahoma City, Oklahoma

THE OCCURRENCE of *Microsporum canis* Bodin ringworm in cats and dogs is of interest to veterinarians and medical practitioners alike.[1]

This fungus, also known in the literature under the names *Microsporum caninum* Sabouraud, *Microsporum felineum* Mewborn, and *Microsporum lanosum* Sabouraud, is over the greater part of the United States the most common cause of tinea capitis in children. When cases in children occur there is usually a clear history of an initial contact with an infected kitten or puppy. What to do about the infected pet then becomes a problem. Where expensive pedigreed animals are involved, the problem may assume some importance economically. Dr. L. K. Georg, of the Communicable Disease Center Laboratories, United States Public Health Service, Chamblee, Ga., has recently brought to the writers' attention one instance in which examination under ultraviolet light disclosed *M. canis* infection widespread and apparently chronic among the pedigreed cats of one breeder. Wider use of this diagnostic tool would doubtless add more examples of the same kind.

For these reasons, the writers are led to believe that a description of the natural course of *M. canis* infections in kittens, observed under laboratory conditions, might have general usefulness at this time.

METHODS

The observations reported here were made during studies of antimycotic agents in the treatment of ringworm. Primarily, the animals used in this report are the untreated controls of these experiments together with animals acquiring accidental infection with *M. canis* in the laboratory. The universal failure of therapeutic agents in ringworm doubtless would have justified larger mention of treated animals as well.

Litters of kittens, 4 to 6 weeks old, were obtained from private owners by placing an advertisement in the newspaper. Only animals which appeared healthy were accepted. After they were received, the kittens were dusted with 5 per cent rotenone powder to eliminate fleas, regularly examined and treated for ear mites, using ether and 5 per cent rotenone in peanut oil, and screened under ultraviolet light for pre-existing *M. canis* infection. There were occasional outbreaks of conjunctivitis which were effectively treated with 1 per cent aureomycin ophthalmic ointment. The kittens were housed in wire cages, 2 to 5 per cage, provided with newspaper bedding and, for elimination, sawdust-filled pans. They were fed fresh frozen ground horsemeat (Hill's Packing Co.) and evaporated and powdered milk, *ad libitum*, supplemented twice weekly with fresh pork liver, frozen whiting, and cod liver oil. All kittens remained healthy, content, playful, and gained weight rapidly during the course of the experiment.

Five kittens were inoculated with the dermatophytes *M. canis* and *Trichophyton mentagrophytes* (granular form) by plucking and abrading 4.0-sq. mm. areas of skin, and rubbing 0.2-sq. mm. bits of agar culture mats of the fungi into the lesions. An inoculation with *T. mentagrophytes*, strain 1A3, was made on the right side, and an inoculation with *M. canis*, strain 2A4, on the left side of each kitten. Seventeen kittens were inoculated with *M. canis* at two sites on each side of the body by the same procedure except that the sites were not scarified, and the inoculums consisted of fluorescent infected hairs obtained from a kitten previously inoculated with strain 2A4.

The progress of the disease was recorded by (1) measuring the diagonal width of the lesions at frequent intervals, (2) recording the proportion of fluorescent hairs observed under ultraviolet light, and (3) culturing plucked hairs and scales on Sabouraud's dextrose agar slants. Intradermal tests for cutaneous sensitivity were made after infection was well established, using 0.1-ml. amounts of 1:100 and 1:10 dilutions of commercial trichophyton as well as of antigens from *M. canis*, strain 2A4, and *T. mentagrophytes*, strain 1A3, prepared by the following methods: (*a*) grinding the acetone-washed culture mat and eluting it quantitatively with propylene glycol, and (*b*) concentrating the culture filtrate in sausage casing with an air fan at 10° C. until the point of precipitation was reached. Observation of the lesions was facilitated by clipping the hair from the sides of each cat with electric clippers and observation of the intradermal tests by shaving the test areas.

Twenty male albino guinea pigs, 2 months old,

From the Department of Dermatology and Syphilology and Department of Bacteriology, University of Oklahoma School of Medicine, Oklahoma City.

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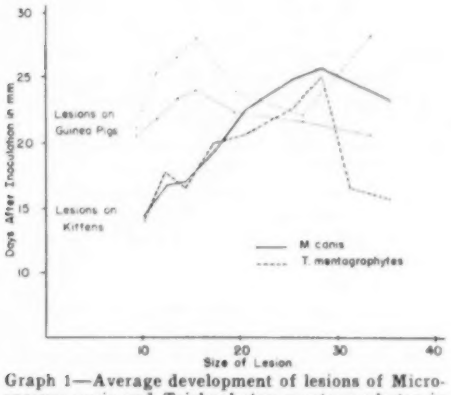
were inoculated with *M. canis* culture, strain 2A4, and *T. mentagrophytes* cultures, strain 1A3, according to the procedure described for the first group of kittens but one year prior to this experiment. The progress of the disease was recorded by the techniques described except that test areas were plucked rather than clipped.



Graph 1—Average development of lesions of Microsporum canis and Trichophyton mentagrophytes in guinea pigs and in kittens in the absence of typical invasion of the hair by M. canis.

Because of a high contamination of the air with viable spores, new kittens and other susceptible animals brought into the experiment room soon became infected with *M. canis*. All kittens, infected and noninfected, were housed in the same room, under identical conditions, in cages side by side. A total of 17 kittens and 10 puppies were accidently infected in this manner prior to experimental inoculation.

Experimental Results

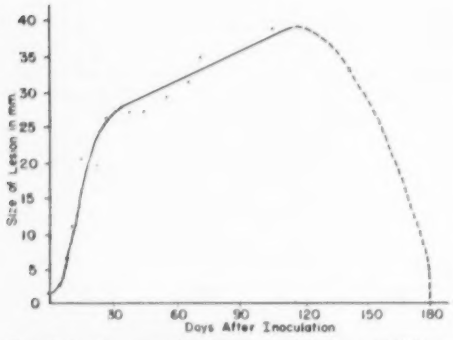
In none of the 28 kittens infected with material from agar slant cultures were fluorescent hairs observed in the lesions developing at the sites of inoculation with *M. canis*, strain 2A4. Microscopic examination of hair from these lesions, which in other respects appeared to be typical, revealed that only minimal invasion of the hair had occurred. Occasional hyphae and arthrospores were seen but no sheath of arthrospores on the surface of the hair. In the lesions resulting from inoculation with *T. mentagrophytes*, strain 1A3, it was found that affluorescent parasitism of the hair, typical of this fungus, had occurred with the production of arthrospores in ectothrix chains. The development and course of the lesions in this group of kittens and in guinea pigs included for comparison is shown (graph 1). The lesions of both *M. canis*, strain 2A4, and *T. mentagrophytes* in kittens were observed to progress through the following three stages: (1) a latent period, lasting about four days after inoculation, before the appearance of a developing spot of erythema, induration, and scaling, signaling the "take" of the infection, (2) a period of rapid growth of the lesion, continuing until about the twenty-eighth day after inoculation, and (3) the regression of the lesion and disappearance of viable fungi (table 1), requiring about ten days longer. Cutaneous sensitivity could not be demonstrated by skin tests. The lesions on the guinea pigs followed essentially the same pattern as those on the kittens (*see* also DeLamater and Benham[2]), with the following exceptions: (*a*) the stage of active development of the lesions lasted about 15 days instead of 28 days, (*b*) the lesions were accompanied by the development of demonstrable cutaneous sensitivity to trichophytin and comparable antigen preparations from the specific fungus strains used, and (*c*) the infections elicited a more marked inflammatory response. Hair invasion and fluorescence occurred. In the case of the guinea pigs the lesions produced by *M. canis* appeared to be somewhat more inflammatory than those produced by *T. mentagrophytes*, whereas in the case of the kittens the reverse was true. The lesions of *M. canis* in this group of kittens and in all of a total of 76 infected kittens, treated and untreated, observed during the course of these studies were

TABLE 1—Record of Cultures of Trichophobic Lesions of Microsporum Canis, Strain 2A4, and Typical Lesions of Trichophyton Mentagrophytes Inoculated Simultaneously in Kittens

Days after inoculation							
11	14	22	27	32	36	41	47
M. CANIS LESIONS							
M*	M	O	O	O	O	O	O
M	M	M	X	O	M	O	O
M	M	M	M	M	O	O	O
M	M	M	M	M	O	O	O
M	M	M	X	O	O	O	O
T. MENTAGROPHYTES LESIONS							
T	T	T	X	T	O	O	O
T	T	T	T	T	O	T	T
T	T	T	T	T	T	T	T
T	T	T	T	O	T	O	O
T	T	T	T	O	T	O	O

* M—*M. canis* obtained; T—*T. mentagrophytes* obtained; X—contaminated cultures; O—no fungus obtained.

essentially noninflammatory in type, *i.e.*, only a slight inflammatory reaction consisting of erythema, slight induration, light scaling, and occasionally slight crusting was produced.

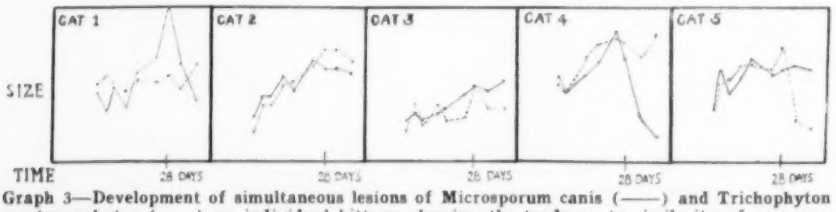


Graph 2—Average development of lesions of Microsporum canis in kittens in the case of typical invasion of the hair.

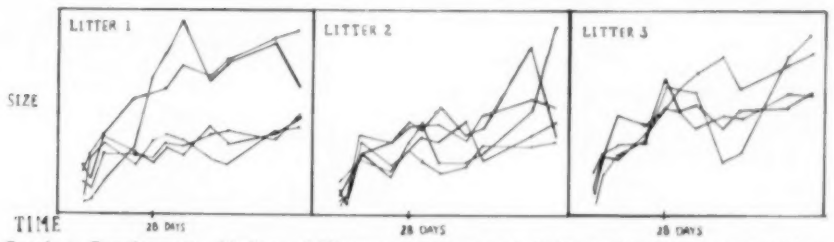
In all of the 17 kittens inoculated with hairs infected with *M. canis*, the hair fluoresced abundantly in the developing lesions. Microscopic examination of fluorescent hairs revealed the ectothrix spores of the fungus in large numbers. The development and course of the lesions in this group of kittens (graph 2) for the first 28 days after inoculation, followed a course substantially the same as that described in the previous experiment. However, the twenty-eighth day of infection was not followed by relatively prompt resolution of the lesions. Instead the lesions tended to increase slowly in size, while at the same time the fluorescent hairs tended to thin out. It was not until five months after infection that final resolution of the infections was definitely in view and at six months there remained only a few fluorescent hairs on a few cats.

The accidental infection of kittens in the laboratory provided an interesting comparison with the experimental infections under study. The accidental infections of previously uninfected kittens most often occurred on the feet, nose, and ears, perhaps because of the rubbing to which these members were subject during the kittens' normal activity and because the short hair of these parts permitted the infecting spores to reach the skin more easily. All of the 17 kittens accidentally infected prior to experimental infection developed the primary lesions on the nose, ears, or feet. On the other hand, none of the 76 experimentally infected kittens, treated and untreated, developed satellite or accidental infections on the nose, ears, or feet, although numerous secondary lesions developed on the areas of skin regularly clipped, particularly at the sites of slight abrasions produced by the clippers. As in the case of the kittens in which the primary lesions followed experimental inoculation, the primary lesions of kittens accidentally inoculated developed a size of several centimeters. Similarly, subsequent lesions, both accidental and experimental, failed to attain large size.

No allergic reaction was obtained by skin testing either the experimentally inoculated kittens or 7 yellow-haired, fair-skinned kittens in the group which acquired accidental laboratory infection. However, the rather sudden and apparently lasting reversal of the course of the lesions on about the twenty-eighth day after inoculation suggested that some sort of cutaneous immunity developed. In each instance after the primary lesion was established, subsequent inoculation, either experimental or accidental, any place on the skin surface failed to develop into large lesions. Such secondary lesions rarely exceeded 1 cm. in diameter, tended to be



Graph 3—Development of simultaneous lesions of Microsporum canis (——) and Trichophyton mentagrophytes (- - - -) on individual kittens, showing the tendency to similarity of response.



Graph 4—Development of lesions of Microsporum canis on individual kittens in separate litters, showing tendency of similarity of response within each litter.

oval in shape, following the fold lines of the skin, exhibited fluorescent hair, and were rather more markedly indurated than the primary lesion. Scaling and crusting of the lesions was most evident for a few days near the climax at the twenty-eighth day of the infections.

Multiple lesions both of the same and of different species on the same kitten followed a pattern of development distinct for each individual animal (graph 3), a situation that seems to be explicable only by postulating physiological factors, capable of subtle changes, which influence the growth of the parasite on the skin. The kittens in certain of the litters responded in a rather uniform fashion to experimental infection, indicating possibly inherited differences in host response (graph 4).

Ten hound dog puppies, 2 months old, housed in the experiment room, developed lesions of *M. canis* promptly. However, in contrast to the lesions observed on the kittens, those on the dogs were indurated and crusted and, in two instances, developed into typical kerions. The lesions were acute and of less than a month duration. Skin tests were not done. However, dogs are known to react to antigens of this kind.

Discussion

The striking contrast between the time required for the resolution of *M. canis* infections produced by inoculums of infected hair and that for the resolution of infections produced by a laboratory culture of *M. canis*, strain 2A4, suggests that in the first case, which is presumably an example of the usual disease, regression of infection following the 28-day-period of rapid growth was prevented by the persistence of viable fungi in the hair, from which station its gradual spread to previously uninfected hair was still possible. The chronicity of *M. canis* infections in the cat may depend, therefore, on the rate of involvement of new hairs relative to the rate of loss of previously infected hair, during this prolonged regression stage. It is possible that the loss of infected hairs may be accomplished primarily by normal shedding. However, in the later stages of the infections observed, hairs were found which fluoresced only at the tips. Apparently these ceased to be infected while still growing *in situ*.

Failure of the 2A4 culture of *M. canis* to parasitize the hair was attributed to attenuation of the strain during its maintenance *in vitro*. This presumptive attenuation was not associated with any observed changes in the morphology of the culture. However, Georg has demonstrated a phenomenon of this kind normally relating the downy and granular forms of *T. mentagrophytes*.[3] Two of the kittens infected with 2A4 culture mat developed fluorescent lesions at sites apart from the sites of inoculation. It is not known whether these lesions originated from the 2A4 strain or from an overlooked exogenous infection. They followed a chronic course, although the primary, nonfluorescent lesions disappeared soon after the twenty-eighth day. Infected hair from the fluorescent lesions of 1 of these kittens served as the inoculum for subsequent experiments, in which fluorescence was regularly observed from the onset of the lesions four days after inoculation. The identical inoculation of 10 cats one year previous to the experiments reported here, as well as of the 20 guinea pigs which have been included, using cultures of the same fungi (strain 2A4, and 1A3) resulted in typical fluorescence and, in the kittens, chronic *M. canis* infections

in the presence of lesions of *T. mentagrophytes*. It does not seem probable, therefore, that in the present instance the Trichophyton lesions established a dominant pattern of immunity, modifying the 2A4 culture lesions as described.

The chronic and relatively noninflammatory character of *M. canis* infections in the cat may indicate that the fungus is specifically adapted to the cat as a host. In man, the zoophilic dermatophytes *M. canis*, *T. mentagrophytes*, and *Trichophyton verrucosum*, tend to produce more inflammatory and transient infections than do the homophilic species *Microsporum audouini*, *Trichophyton tonsurans*, and *Trichophyton rubrum*. The ability of the homophilic species to persist in a chronic state without producing an inflammatory reaction or marked cutaneous sensitivity may have survival value and be part of their adaptation to the special parasitism of man. The *M. canis* infections we have observed in kittens resemble *M. audouini* infections in man more than they do the more inflammatory infections usually produced in man by *M. canis*.

SUMMARY

The course of experimental and accidental laboratory infection of cats with *Microsporus canis* is described. The lesions were observed to develop about four days after inoculation, increase rapidly in size for about 28 days and then enter a phase of regression. The chronicity of the lesion was found probably to be dependent on the persistence of the fungus pathogen in the hair. In the absence of hair invasion, an atypical situation dependent on the use of an apparently attenuated strain of *M. canis*, resolution of the infection was found to occur about one month after inoculation. However, when hairs were infected the infection was observed to require six months on the average to disappear entirely, depending somewhat upon the individual cat and possibly upon the rate of loss of infected hairs through the agency of natural shedding.

Once an initial infection was established (*i.e.*, had run a substantial part of its 28-day-course of rapid growth) subsequent test infections failed to develop into large lesions and the animal was relatively immune to natural infection which occurred principally on the feet, nose, and ears. However, under these circumstances artificial inoculation was still possible and the infection was still spread by clipping the hair, with its attendant slight trauma to the skin.

Infections of *M. canis* in kittens were observed to produce only minimal inflammatory response which characteristically reached its peak near the twenty-eighth day climax. No skin test reaction was elicited by trichophytin and antigen preparations from the fungus strains used, the potency of which was demonstrated when tested on infected guinea pigs and on man. The accidental infection of 10 hound dog puppies with *M. canis* produced indurated and crusted lesions and in two instances a kerion. It is suggested that in view of the chronicity of infections and the minimal inflammatory response produced in cats, that cats provide the major reservoir and probably the "natural" host of the fungus in *M. canis*.

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Effects of the Glycemic Levels on Rumen Motility in the Sheep

G. AUGUSTO VALLENAS P., D.V.M., M.S.

Ithaca, New York, and Lima, Peru

EVIDENCE has been obtained to indicate that carbohydrate metabolism is in some way correlated with gastric motility in the animal with a simple stomach.

Bulatao and Carlson[1] in the dog, Mulinos[2] in the dog, Quigley, Johnson, and Salomon[3] in the dog and in man, and many others, have reported that under certain conditions insulin hypoglycemia is accompanied by an increase in the gastric hunger contractions. Bulatao and Carlson[1] reported that stomach motility in the dog could be depressed by the intravenous administration of strong glucose solutions.

Products of carbohydrate breakdown in the rumen play an important role in intermediary carbohydrate metabolism of ruminants, not only in the physiological state but also under pathological conditions. A question arose as to whether the variation in blood sugar level would affect the rumen motility in sheep in the same way as in man and in dog. To elucidate the problem and in the hope that additional information might be gained concerning the physiological factors affecting rumen motility, these experiments were devised and carried out.

While the experiments were in progress, Le Bars *et al.*[4] reported that induced hyperglycemia exerts an inhibitory effect on the rumen motility in sheep and that hypoglycemia on the other hand elicits hypermotility.

This work, therefore, deals with confirmation of the results obtained by the French authors, and also with additional data that are considered to be of interest.

MATERIAL AND METHODS

Seven Western sheep, which included 5 males and 2 females, were employed in these experiments. They varied in age from 1 to 3 years and in weight from 67 to 137 lb. They were fed a standard ration consisting of hay and grain and water *ad libitum*. An effort was made to minimize environmental stress, and particularly excitement, by placing another sheep in proximity to the experimental animal during the period in which the experiment was in progress.

A permanent closed rumen fistula was made in all sheep by surgically placing a rumen cannula in the left paralumbar fossa region and allowing the wound to heal.[5] The rumen cannula-fistula was connected directly with a tambour (fig. 1). A rubber stopper in the external opening of the cannula served to close the ruminoreticular cavity and to connect the whole structure to the rubber tubing-tambour system. The tambour was provided with a writing point which recorded the waves of rumen contraction on a continuous-feed ink-writing kymograph. In all instances, the time was recorded simultaneously.[6]

In view of the great variability of rumen motility in frequency as well as in amplitude in normal sheep,[7, 8] a tracing was taken for a period of one to two hours before each injection in order to get a representative picture of normal rumen motility. Later, motility recordings were made during injections of physiological saline to determine if the process of intravenous injection influenced rumen motility.

All solutions were administered intravenously. In some experiments, the solution was infused by means of a venoclysis continuous-drip apparatus connected with a small-diameter polyethylene tube inserted in the left jugular vein.

Five-milliliter samples of blood were drawn from the jugular vein prior to and following the various injections. A mixture of potassium oxalate and sodium fluoride was used as the anticoagulant. Blood glucose was determined with the method of Schaffer and Somogyi.[9] The procedure described by Roe[10] and modified by Higashi and Peters[11] was adopted to determine the blood fructose level using the Somogyi filtrate. Concomitant with the fructose level determination, the glucose level was calculated by first determining the total hexose content in the Somogyi filtrate[9] and then subtracting the fructose level.

To evaluate the changes of frequency and amplitude of rumen contractions associated with the injection of glucose, insulin, or fructose, the following procedure was adopted. All the tracings composed of the preinjection and postinjection recordings were divided into 20-minute periods; the number of contractions in each period was counted and considered as representing that 20-minute period. As to the preinjection frequency of contraction, the mean calculated from several 20-minute periods was taken as representing the preinjection normal frequency. The postinjection frequency value was obtained in the same manner. In order to obtain the preinjection amplitude of contraction, the mean amplitude value of each 20-minute period was averaged, and the value thus obtained was considered as representing the pre-

From the Department of Physiology, New York State Veterinary College, Cornell University, Ithaca.

This is a condensation of a thesis presented to the faculty of the Graduate School of Cornell University in September, 1954, in partial fulfillment of the requirements for the degree of Master of Science; major professor, Dr. R. W. Dougherty.

Dr. Vallenas is associate professor of Veterinary Physiology in the Faculty of Veterinary Medicine, National University of San Marcos, Lima, Peru.

injection normal amplitude. Similarly, the amplitude value for each 20-minute period following the injection was obtained by taking a mean of amplitudes for that period. These precautions were taken in order to eliminate the possibility of spontaneous changes being accepted as the action of a substance under test.

When a method of counting the number of rumen contractions on the tracings was being devised, the question arose as to whether all waves recorded represented major rumen contractions. Every second, third, or fourth wave recorded was smaller than its predecessor and was usually associated with a slight diminution in the intraruminal pressure. Each of these smaller waves generally coincided with an eructation and did not represent a major rumen contraction. The smaller waves were attributed, therefore, to events associated with eructation and consequently were not counted among the major rumen contractions. The number of rumen contractions counted was so large that it is believed that the elimination of these waves did not materially affect the significance of the results obtained and conclusions drawn.

RESULTS

Effect of the Intravenous Injection of Glucose on Rumen Motility.—Five sheep were injected through the jugular vein with a 50 per cent solution of glucose. All animals were again subjected to the same experiment after a week's interval. The doses of glucose varied from 0.3 to 1.0 Gm. of glucose per kilogram of body weight.

A sheep that was injected with 0.3 Gm. of glucose per kilogram of body weight showed no significant change in rumen motility.

Another sheep was injected with 0.7 Gm. of glucose per kilogram of body weight (graph 1). The amplitude and frequency of rumen contractions diminished immediately after injection. The amplitude diminished to 75 per cent of the normal value 100 minutes after injection. The frequency fluctuated, dropping to 84 per cent of the initial value 160 minutes after the injection. As the blood glucose level returned to normal, the motility gradually became normal.

Each of 4 sheep received 1.0 Gm. of glucose per kilogram of body weight. The characteristic results (graph 2) include a primary diminution of amplitude and frequency of rumen motility soon after the glucose injection. This period was followed by a slight increase in frequency as well as in amplitude, after which the frequency continued to decline until the contractions were no longer perceptible 100 minutes after injection. The diminution of amplitude nearly paralleled that of frequency.

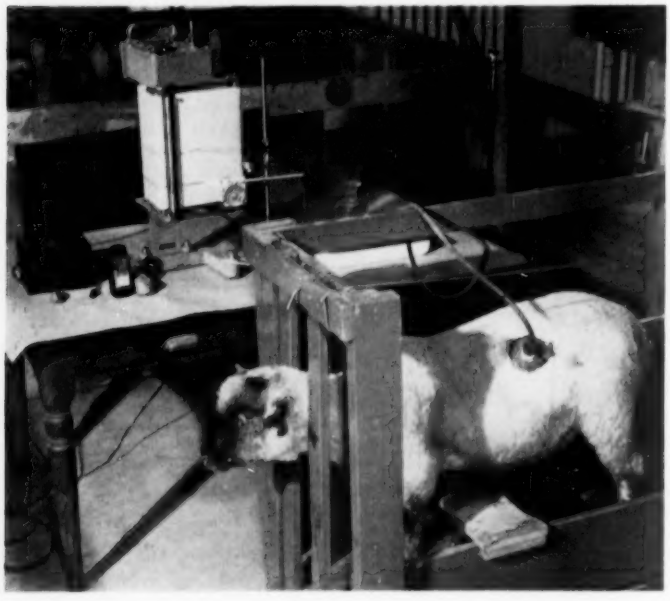
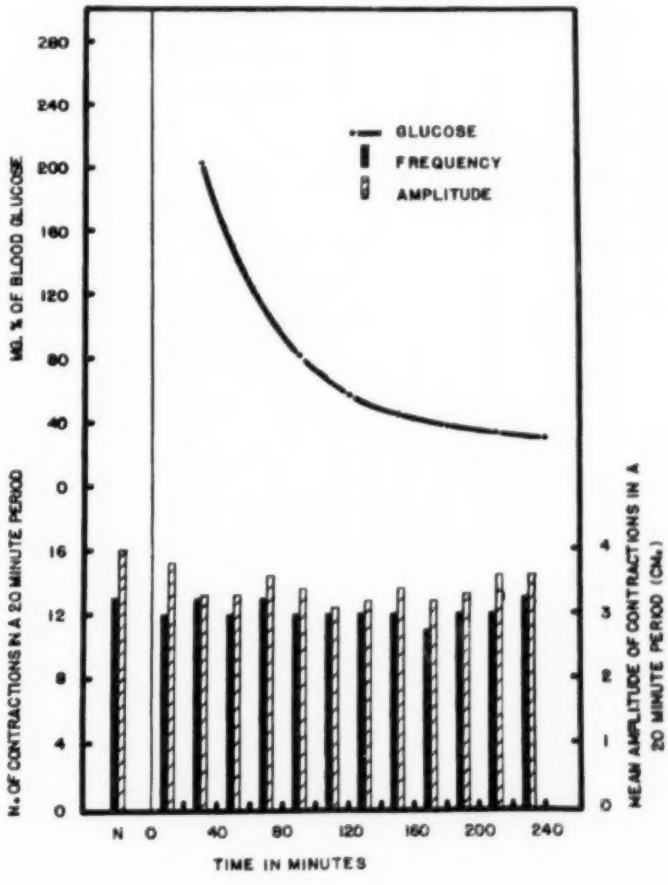


Fig. 1—Arrangement of the apparatus for recording rumen contractions.

After 120 minutes the frequency and the amplitude started to increase, while the blood glucose nearly reached its normal value.

To summarize, small doses of glucose, 0.3 to 0.7 Gm. per kilogram of body weight, produced only a slight effect on the amplitude and little or no effect on the frequency of rumen contractions. With a dose of 1.0 Gm., the effect in all sheep was a marked reduction in frequency as well as in amplitude of the contractions. The intensity of this effect varied from one sheep to another and from one day to another, although the experimental conditions were maintained as constant as possible. There was a wide variation even in duplicate determinations made in the same individual. After reaching the lowest level, usually 100 to 140 minutes after injection, both amplitude and frequency began to rise and return to the normal level 220 to 240 minutes after the injection. This period coincided approximately with the fall of the glucose level to normal.

Effect of the Intravenous Injection of Insulin on Rumen Motility.—Five sheep were used to study the effect of induced hypoglycemia on rumen motility. All were used twice with a week between experiments. The dose of insulin (iletin) injected into the jugular vein was 1 unit per kilogram of body weight, the amount known to produce a maximum depression of blood glucose for a desired period.[12] The results (graph 3) show the characteristic

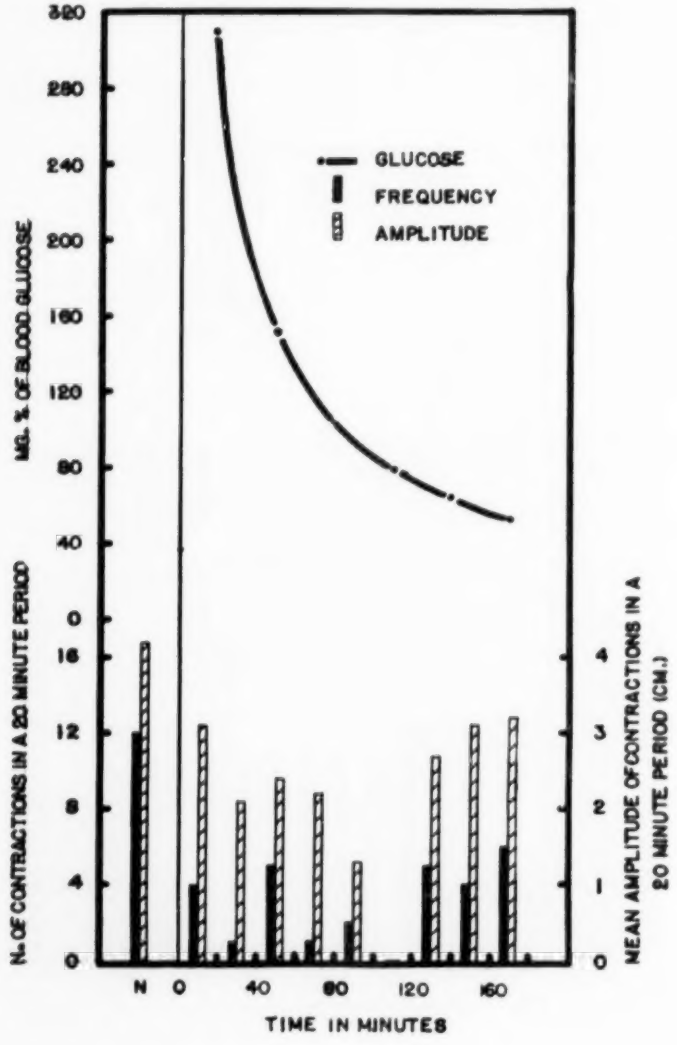


Graph 1—Effect of glucose injection, 0.7 Gm. per kilogram of body weight, on rumen motility.

N = preinjection normal values of blood glucose as well as of frequency and amplitude of rumen contractions; 0 = time of injection.

effects of induced hypoglycemia on rumen motility, an immediate diminishing after insulin injection, then both the frequency and amplitude increased, until they were above the normal level. Thus, 100 minutes after injection the frequency had increased over 50 per cent and the amplitude 15 per cent above the normal level. After this period, the amplitude gradually returned to approximately the normal level, while the frequency, though declining, remained far above the normal level.

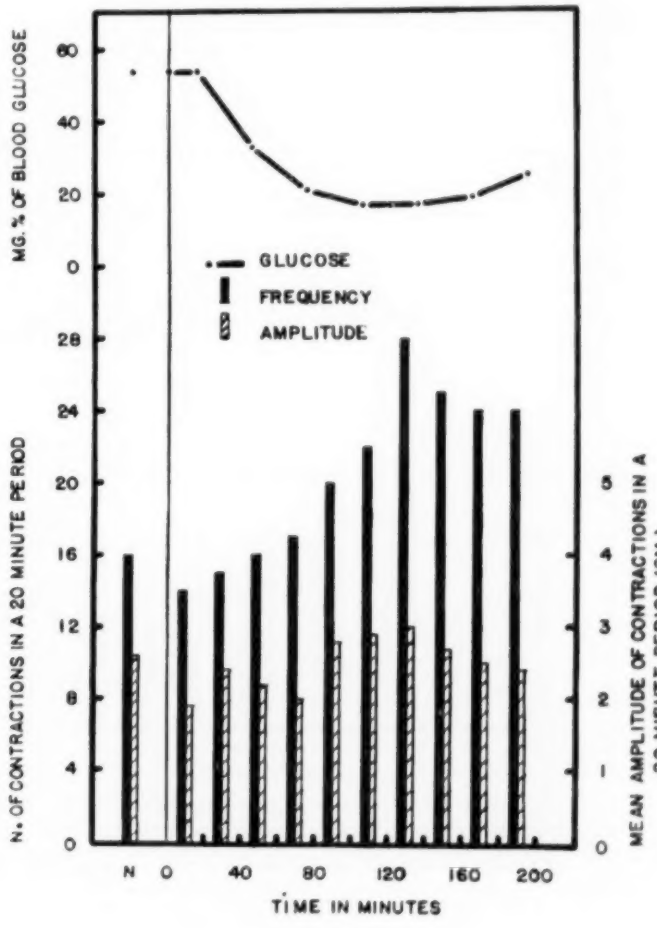
In general, insulin produced a transitory inhibitory effect on rumen contractions immediately after the injection, which was followed by an increase in frequency and amplitude, the maximum occurring 100 to 160 minutes after the injection. An interesting observation was that each of the sheep started to ruminate at this time. After this period, the motility resumed the normal pattern, as the glycemic level gradually returned to normal. In most of the experimental animals



Graph 2—Effect of glucose injection, 1 Gm. per kilogram of body weight, on rumen motility. N = preinjection normal values of blood glucose as well as of frequency and amplitude of rumen contractions; 0 = time of injection.

the return to a normal level of glycemia required more than 240 minutes. There appeared to be no constant relationship between the onset of hypoglycemia and of hypermotility. Individual sheep differed in their response to insulin; the amplitude and frequency did not always vary simultaneously; that is, slight increase in amplitude was, in some instances, accompanied by a greater increase in frequency and vice versa (graph 3). Certain sheep manifested symptoms such as trembling and shivering, when the lowest hypoglycemic level was reached.

Effect of the Injection of Glucose on Rumen Hypermotility Associated with Hypoglycemia.—Experiments were undertaken to observe the effect of glucose injection on such hypermotility of the rumen. Following the injection of insulin in a dose of 1 unit per kilogram of body weight, a stimulatory effect on rumen motility ensued, as observed in the previous experiment. While this effect was still maintained, a glucose solution, 1 Gm. per kilogram of body weight, was injected with consequent decrease of 50 per cent in frequency and 65 per cent in amplitude of the normal values about 60 minutes after the injection of glucose (graph 4, 5). Thus it appears that the hypermotility produced by insulin injection was counteracted by the inhibitory effect elicited by intravenous injection of glucose.

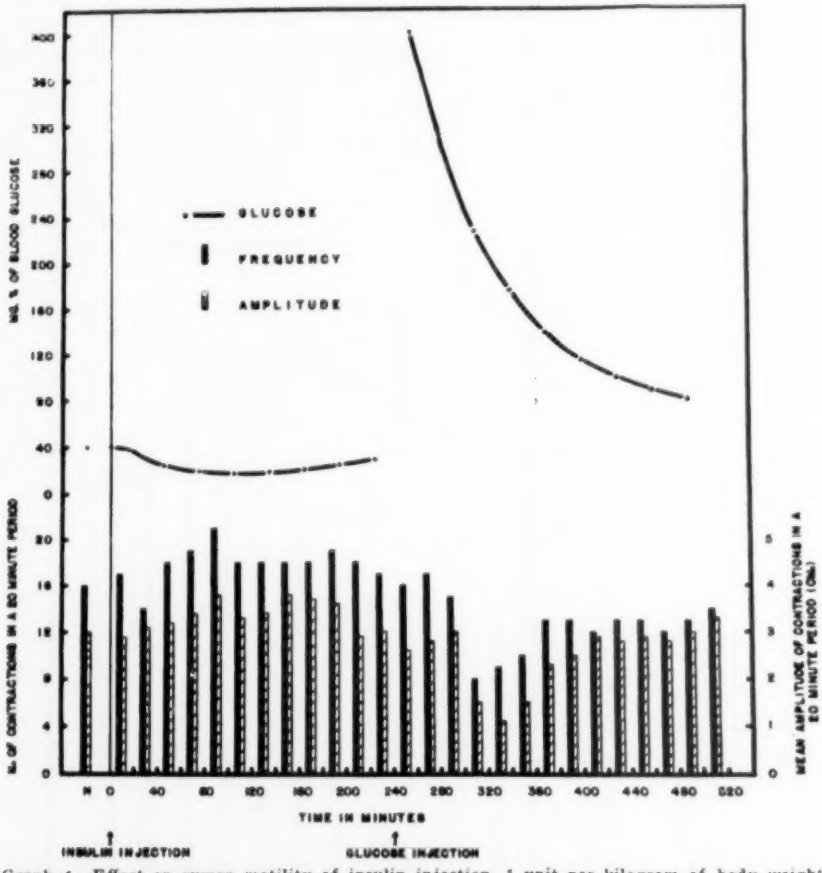


Graph 3—Effect of insulin injection, 1 unit per kilogram of body weight, on rumen motility. N = preinjection normal values of blood glucose as well as of frequency and amplitude of rumen contractions; 0 = time of injection.

Effect of the Infusion of Fructose on Rumen Motility.—It was desirable to ascertain the possible effect of a sugar other than glucose on rumen contractions. Therefore, a fructose solution was used because of the rapid disappearance of this sugar from the blood as has been shown by other authors.[15]

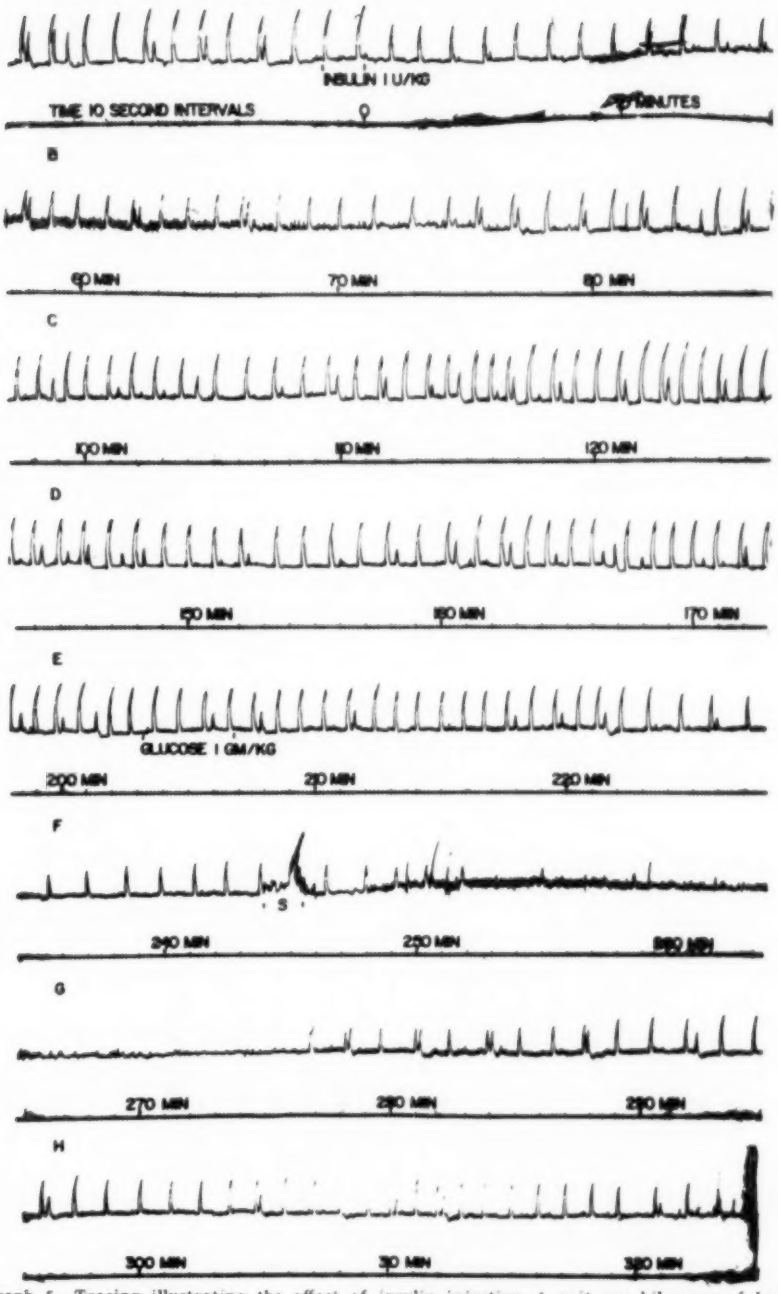
Five hundred milliliters of a 20 per cent fructose solution were infused at a rate varying from 1.5 ml. to 3.1 ml. per minute (graph 6). The frequency of the rumen movements was unaltered during the first 40 minutes of infusion while the amplitude increased slightly. At the end of 80 minutes, the frequency of rumen contractions had diminished to 7 per cent and the amplitude to about 30 per cent of the normal level. After this period, both frequency and amplitude increased slightly again, although remaining far below the normal level. The rumen contractions disappeared completely for a period of 40 minutes at approximately 160 minutes after the start of infusion. During the next 20 minutes they were very low, after which they increased markedly, rising to slightly above the normal levels.

Different blood levels of fructose and glucose are also plotted (graph 6). The


MG. % OF BLOOD GLUCOSE
GLUCOSE
FREQUENCY
AMPLITUDE
NO. OF CONTRACTIONS IN A 80 MINUTE PERIOD
MEAN AMPLITUDE OF CONTRACTIONS IN A 80 MINUTE PERIOD (CMS.)
N 0 40 80 120 160 200 240 280 320 360 400 440 480 520
TIME IN MINUTES
INSULIN INJECTION
GLUCOSE INJECTION

Graph 4—Effect on rumen motility of insulin injection, 1 unit per kilogram of body weight, followed in four hours by glucose injection, 1 Gm. per kilogram of body weight. N = pre-injection normal values of blood glucose as well as of frequency and amplitude of rumen contractions.

Rumen Contractions



Graph 5—Tracing illustrating the effect of insulin injection, 1 unit per kilogram of body weight, followed by glucose injection, 1 Gm. per kilogram of body weight, on rumen motility. S — time blood sample was drawn.

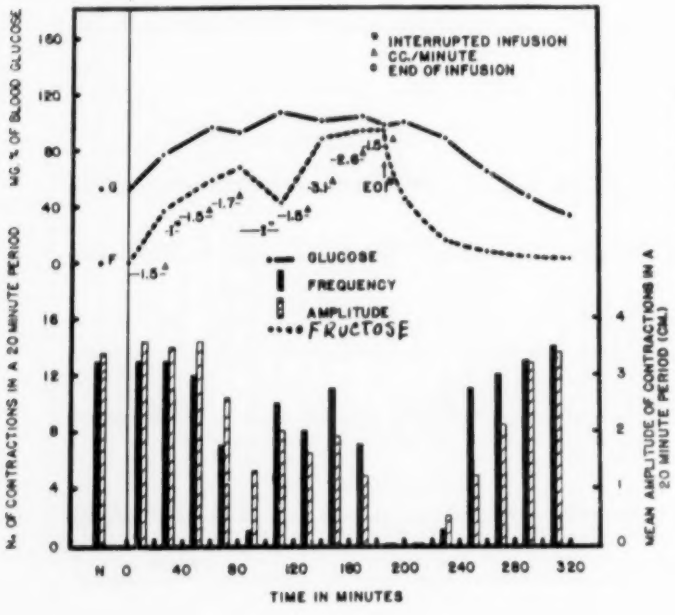
fructose level rose throughout the period of infusion; the drop observed in the curve is probably due to an accidental interruption of infusion period. Discontinuation of fructose infusion resulted in a rapid drop of fructose level. The rise in the blood glucose level nearly paralleled that of fructose, although a more gradual decline of the glucose curve as compared with fructose gave rise to a longer period of hyperglycemia after the cessation of infusion.

Effect of the Injection of Fructose on Rumen Motility.—To ascertain whether a single injection of fructose would produce the same effect as does infusion, a 50 per cent fructose solution of 1 Gm. per kilogram of body weight was injected into the jugular vein of each of 2 sheep (graph 7). Immediately after injection of the fructose, there occurred a slight inhibition in frequency as well as in amplitude for about 120 minutes, followed by a slight rise above the normal level. In addition, blood fructose and glucose levels were determined and plotted. It is evident that the disappearance of fructose was rapid but that its injection affected the glucose level, producing an increase followed by a diminution.[13] Also, the period of inhibition of rumen motility corresponded to the hyperglycemic level and the slight acceleration of motility to the relative hypoglycemia.

Effect of the Injection of Saline Solution on Rumen Motility.—To eliminate the possibility that stimuli caused by any injection would produce changes in rumen motility, 1 sheep was injected in the jugular vein with 50 ml. of a sterile saline solution. Tracings were taken for an hour both before and after the injection. There was no appreciable variation in the frequency and amplitude of rumen contractions.

Discussion

Injection of glucose in a dose of 1 Gm. per kilogram of body weight produced inhibition of rumen motility, and intravenous injection of insulin, 1 unit per kilogram of body weight, provoked hypermotility of the rumen. This was observed in all sheep. The results obtained confirm the observations of previous investigators.[1-4] It is of interest that the effect of injected sugar or insulin on rumen motility becomes apparent only after a certain period. The amplitude and frequency did not al-

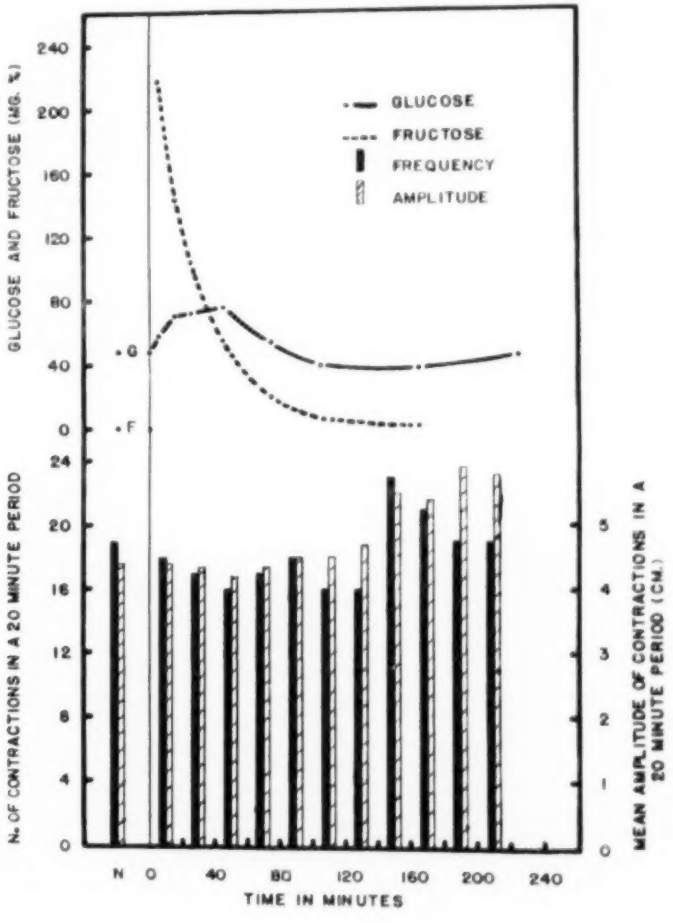


Graph 6—Effect of fructose infusion on rumen motility. N = preinjection normal values of blood glucose (G) and fructose (F) as well as of frequency and amplitude of rumen contractions; O = start of infusion.

ways vary simultaneously; decrease in the frequency of the rumen contractions occurred in some instances, these being more powerful as evidenced by a greater amplitude.

It is not clear whether any correlation exists between blood sugar concentration and the onset of the stimulation or inhibition of the rumen contractions. There are wide variations even in duplicate determinations in the same animal. Moreover, the impression gained from the data is that no predictable correlation exists between blood sugar levels and the time of onset, or the degree of rumen hypermotility or hypomotility. The onset of the hypermotile (insulin) or hypomotile (glucose) phase is gradual rather than abrupt, and it does not terminate abruptly even when the normal glycemia is restored. The duration of hypermotility due to insulin injection seems to be variable, but it may apparently outlast the hypoglycemic period.

Hypermotility due to induced hypoglycemia may be compared to hunger contractions observed by many authors in animals with a simple stomach.[14, 15] It has been observed that in man, and probably in carnivorous animals, an empty stomach is a requisite for the appearance of hunger contractions. This is evidently not the case in ruminant animals because the stomach



Graph 7—Effect of fructose injection, 1 Gm. per kilogram of body weight, on rumen motility. N = preinjection normal values of blood glucose as well as of frequency and amplitude of rumen contractions; 0 = time of injection.

will not be empty even after 5 or 6 days of starvation.

Immediately following insulin injection a transitory period of inhibition of rumen motility, particularly in amplitude, was observed. It was thought that this primary inhibition, which was also observed in some sheep following glucose injection, might be due to reflex discharge of adrenaline caused by the pain arising from the intravenous injection and by restraining of the sheep. Adrenaline could inhibit rumen motility by way of the sympathetic nervous system.[16,17] However, it was noticed that injection of saline solution produced no alteration in rumen motility; therefore, the primary inhibition could not be attributed to adrenaline discharge.

Regan [18] reported that the intravenous injection of insulin in normal dogs produced a primary depression of tonus and gastric activity which passed over into gastric hypermotility. It was reported also [19,20] that intravenous injection of insulin stimulates the sympathetic nervous system which results, among others, in a decrease in tone and quiescence of the stomach. Scott,[21] working on dogs which previously had been vagotomized, found that the immediate inhibitory action of intravenous insulin on gastric motility is more marked and more frequent than before vagotomy. On the other hand, it is already known [17,22] that in ruminants the splanchnic nerves (sympathetic system) carry impulses that depress gastric motility. Thus, the transitory inhibition which always followed the insulin injection might be attributed to insulin itself and not to the sugar level in the blood.

The nervous system could be affected directly by glycemia, or by some other mechanism involved in the phenomenon. It is assumed that the vagal stimuli originate in some center in the central nervous system, called "ruminal centre" by some authors,[17] or "rumination center" by some others,[22] which discharges a series of regular impulses, much as does the respiratory center, and that it possesses an inherent rhythm influenced by humoral and nervous factors, or else that this center discharges only in response to specific stimuli. Clark [23] made an attempt to localize this center in the subcortical area anterior to the infundibulum. Evidently the relationship existing between the level of glycemia and rumen motility as observed in these experiments is in favor of the hypothesis of the humoral regulation. That hypoglycemic hypermotility was followed by an initiation of rumination is another fact that may support the humoral theory, inasmuch as it is already known that rumen motility is increased during rumination. The variation of the glucose level might affect the central mechanisms which regulate not only the motility of the rumen but also the complex phenomenon of rumination.

Injection of fructose produced slight changes in rumen motility. Following the injection of fructose, the glucose level showed an initial hyperglycemia of short duration followed by a slight fall. It may be, however, that these slight changes in rumen motility are associated with changes of the glucose level and not with fructose itself, inasmuch as infusion of fructose for 190 minutes induced a considerably elevated glucose level that lasted even after the infusion of fructose was discontinued.

Summary

The effects of injections of glucose, insulin, and fructose on rumen motility were studied using 7 sheep with permanent rumen cannula-fistulas.

1) An induced hyperglycemia, due to an injection of 50 per cent glucose solution at a dose of 1 Gm. per kilogram of body weight, provoked an inhibitory effect on rumen motility.

2) An induced hypoglycemia, caused by an intravenous injection of 1 unit of insulin per kilogram of body weight, resulted in a stimulation of rumen contractions preceded by a transitory inhibition period.

3) The induced hypoglycemic hypermotility was inhibited by injection of a 50 per cent glucose solution in a dose of 1 Gm. per kilogram of body weight.

4) Infusion of fructose in an amount of 500 ml. of a 20 per cent solution during a period of 160 minutes caused inhibition of rumen motility.

5) Injection of a 50 per cent fructose solution in a single dose of 1 Gm. per kilogram of body weight produced no apparent effect on rumen motility.

6) The theory that the variations of

blood glucose level may affect the ruminal center by way of a humoral mechanism has been discussed.

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Chemotherapy of Canine Cancer with N-(3-Oxapentamethylene)-N′, N″-Diethylenephosphoramide (MEPA)

JOHN R. McCOY, V.M.D.; J. B. ALLISON, Ph.D.; M. L. CROSSLEY, Ph.D.;
R. W. WANNEMACHER, JR., M.S.

New Brunswick, New Jersey

SOME of the depleting effects of cancer on the "labile protein stores" of dogs have been evaluated. These "stores" are the blood and tissue proteins that are being catabolized continually to supply amino acids and energy for maintenance of essential structures. Some of them are the enzyme systems that detoxify poisons, protect the animal from the toxicity of chemotherapeutic agents, and catalyze reactions needed for resistance to stresses of all kinds. Mixtures of amino acids or of hormones or of both are being sought to reduce the catabolism of normal tissues.

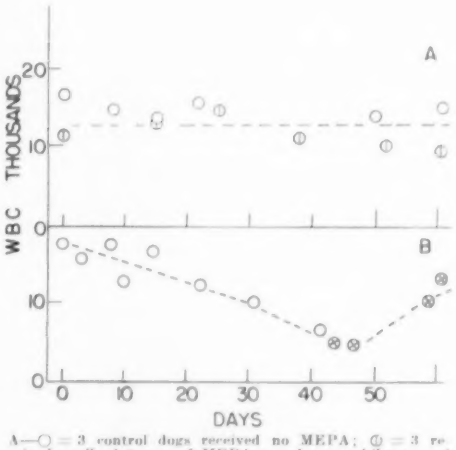
It was reported previously that the addition of methionine to the diet in proper proportions reduced the catabolism of normal tissue proteins in the presence of the tumor, thereby improving the welfare of the animal.[1] This reduction, however, was most obvious during the early growth of the tumor and was not effective after the cancer had begun to markedly deplete normal tissues. It was found that a mixture of glycine and methionine or of glycocyamine and methionine increased the favorable response of methionine supplementation, the mixture of methionine and glycocyamine being particularly effective under conditions of stress such as are associated with marked depletion of "protein stores" or the toxicity of the phosphoramides.[2-6]

The phosphoramides have been used to reduce the rate of growth and even cause regressions in some tumors.[7-11] Preliminary studies indicated that N-(3-oxapentamethylene)-N′, N″-diethylenephosphoramide (MEPA) was of interest in the treatment of naturally occurring tumors in dogs. The following report is restricted to the use of MEPA in the dog.

RESULTS

Effect of MEPA on White Blood Cell Count.—Previous experience demonstrated that the toxicity of MEPA could be measured best by the development of a leukopenia. The data in graph 1 illustrate the average response to giving MEPA in the food to 3 dogs maintained on a low casein diet containing 0.2 Gm. of nitrogen per day per kilogram of body weight. This diet has been described previously.[12] The open circles in graph 1 (A) record the average white blood cell count in 3 control dogs fed this diet without MEPA. The circles with horizontal bars illustrate average data obtained on 3 dogs given orally 1 mg. of MEPA per day per kilogram of body weight. The average white blood cell count was essentially normal in animals given the 1-mg. dosage. The open circles in B, on the other hand, illustrate the leukopenia that developed in 3 normal dogs fed 5 mg. of MEPA per day per kilogram of body weight. When the drug was removed, the count returned to normal (*see* circles with cross bars, graph 1).

The data in graph 2 (C) record the leukopenia which developed in another group



A—○ = 3 control dogs received no MEPA; ⊕ = 3 received orally 1.0 mg. of MEPA per day per kilogram of body weight.
B—○ = 3 received orally 5.0 mg. of MEPA per day per kilogram of body weight; ⊗ = MEPA discontinued.

Graph 1—The average white blood cell response to N-(3-oxapentamethylene)-N′,N″-diethylenephosphoramide (MEPA) in the food of dogs maintained on a low casein diet containing 0.2 Gm. of nitrogen per day per kilogram of body weight.

From the Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

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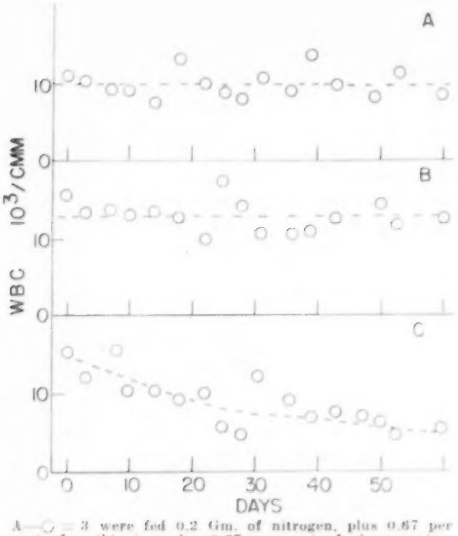


A—○ = 3 were fed 0.2 Gm. of nitrogen, plus 0.67 per cent of methionine, plus 0.67 per cent of glycocyamine, plus 5.0 mg. orally of MEPA per day per kilogram of body weight.
B—○ = 3 were fed 0.6 Gm. of nitrogen, plus 5.0 mg. orally of MEPA per day per kilogram of body weight.
C—○ = 3 were fed 0.2 Gm. of nitrogen plus 5.0 mg. orally of MEPA per day per kilogram of body weight.

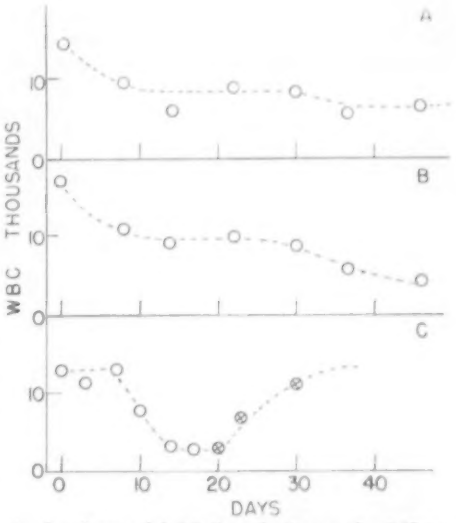
Graph 2—The average white blood cell response to N-(3-oxapentamethylene)-N′,N″-diethylenephosphoramide (MEPA) in the food of dogs maintained on a low casein diet.

of 3 dogs fed 5 mg. of MEPA in the low casein diet. The data in B illustrate the protective effect of increasing the casein nitrogen to 0.6 Gm. per day per kilogram of body weight. Graph 2 (A) records the average white blood cell counts of 3 dogs fed the low casein nitrogen diet supplemented with methionine plus glycocyamine (0.67 % of methionine plus 0.67 % of glycocyamine). Thus, a high casein diet or a supplemented low casein diet prevented the development of leukopenia over this experimental period.

MEPA, injected intravenously, was more toxic than when given orally. The data in graph 3 (C) illustrate the marked leukopenia which developed in 3 normal dogs receiving 1 mg. of MEPA per kilogram of body weight intravenously three times weekly, dogs which were fed 0.2 Gm. of casein nitrogen per day per kilogram. The return to a normal count after cessation of the injections is illustrated by the circles with cross bars. The protective effect of feeding a higher casein diet (0.6 Gm. of nitrogen) is illustrated in B, data being obtained on 3 dogs injected thrice weekly over the whole experimental period with 1.0 mg. of MEPA per kilogram of body weight. The protective effect of methionine plus glycocyamine is illustrated in graph 3 (A), data being obtained on dogs fed the low casein diet supplemented with the mixture of methionine plus glycocyamine and injected with 1.0 mg. of MEPA per kilogram thrice weekly during the entire period of 45 days.

The results (graphs 1, 2, 3) demonstrate that maintenance of the protein stores of the animal is correlated with resistance to development of leukopenia in the presence of MEPA in the dog.

The Effect of MEPA on the Red Blood Cell Count and Hemoglobin.—The red blood cell count and hemoglobin were reduced eventually in dogs given orally 5.0 mg. of MEPA per day per kilogram of

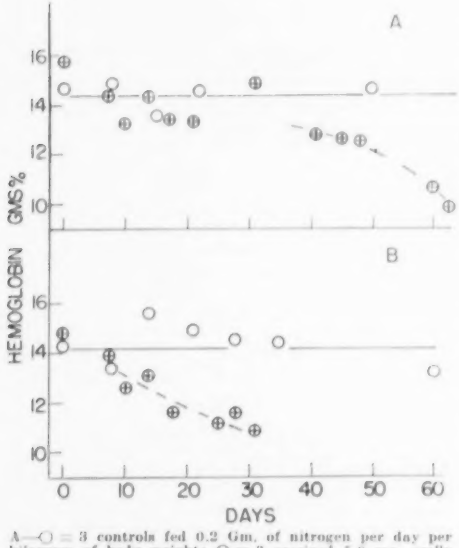


A—○ = 3 were fed 0.2 Gm. of nitrogen plus 0.67 per cent of methionine and 0.67 per cent of glycocyamine per day per kilogram of body weight plus 1.0 mg. of MEPA per kilogram of body weight intravenously thrice weekly throughout the experimental period of 45 days.
B—○ = 3 were fed 0.6 Gm. of nitrogen per day per kilogram of body weight, plus 1.0 mg. of MEPA per kilogram of body weight intravenously thrice weekly throughout the experimental period.
C—○ = 3 were fed 0.2 Gm. of nitrogen per day per kilogram of body weight, plus 1.0 mg. of MEPA per kilogram intravenously thrice weekly for two weeks; ⊗ = MEPA discontinued.

Graph 3—The average white blood cell response to N-(3-oxapentamethylene)-N′,N″-diethylenephosphoramide (MEPA) given intravenously to dogs maintained on a low casein diet.

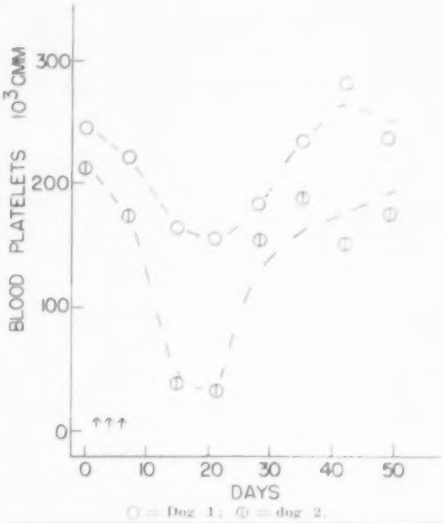
body weight with a diet of 0.2 Gm. of casein nitrogen per day per kilogram (graph 4 A). The open circles in A record the average hemoglobin content of the blood of control dogs fed 0.2 Gm. of casein nitrogen per day per kilogram of body weight. The circles with cross bars illustrate the hemoglobin content of dogs fed the low protein diet plus 5 mg. of MEPA per day per kilogram of body weight. However, toxicity of MEPA can not be detected early through red blood cell counts or through hemoglobin concentration.

In general, the dog will raid the labile protein stores to maintain hemoglobin in the blood.[13] This maintenance of hemoglobin even on a protein-free diet is illustrated by the open circles in graph 4 (B). The greater susceptibility of animals fed the protein-free diet, however, to the toxicity of the drug is illustrated by the circles with cross bars. These animals were given the protein-free diet containing MEPA equivalent to 5.0 mg. per day per kilogram of body weight. Two of these



A—○ = 3 controls fed 0.2 Gm. of nitrogen per day per kilogram of body weight; ⊗ = 3 received 5.0 mg. orally of MEPA per kilogram of body weight.
B—○ = 3 fed protein-free diet; ⊗ = 3 received 5.0 mg. orally of MEPA per kilogram of body weight per day while fed protein-free diet.

Graph 4 — The average hemoglobin response to MEPA in the food of dogs maintained on a low casein diet (A) and a protein-free diet (B).



Graph 5—Blood platelet counts in each of 2 normal dogs fed low casein diet and given three intravenous injections (arrows) of 1.0 mg. of N-(3-oxapentamethylene)-N′,N″-diethylenephosphoramide (MEPA) per kilogram of body weight.

dogs eventually refused food and had to be taken off the diet. Under these conditions, a leukopenia did not develop as rapidly as would be expected, possibly the result of reduced drug intake. Further work should be done, however, to evaluate the relative effect of the diet on the bone marrow.

Effect of MEPA on Blood Platelet Count.—Blood platelets are also quite susceptible to the toxic effects of MEPA (graph 5). These data were obtained on 2 normal dogs with low protein stores maintained on 0.2 Gm. of casein nitrogen per day per kilogram of body weight. Each dog was given 1 mg. of MEPA per day per kilogram of body weight intravenously for three days as indicated by the arrows. The platelets dropped rapidly, the temperatures of the animals increased and they appeared in poor clinical condition. The platelet count increased as the dogs improved and returned to normal. The blood platelet count, however, has not been as good an indication of early drug toxicity in the dog as the white blood cell count.

Plasma Proteins.—The plasma albumin-globulin ratios for the normal dogs under treatment are illustrated in graph 6. The

bar with slanted line over C records the average ratio for 3 dogs fed the low casein diet. This A/G ratio is below the average normal of 0.7 but it is a ratio within the range found for dogs with cancer. The bars over D and E illustrate the average ratios of the dogs fed this amount of casein nitrogen plus 5 mg. of MEPA per day per kilogram of body weight, data which suggest that feeding MEPA tends to deplete plasma albumin still further. The bar over B records the ratio for dogs fed the high casein diet (0.6 Gm. of casein nitrogen per day per kilogram of body weight) plus the MEPA, while the bar over A illustrates the average ratio in these animals fed the low casein diet supplemented with methionine and glycocyamine plus MEPA. Supplementing with methionine or methionine plus glycocyamine reduced the excretion of urea nitrogen, thereby increasing the retention of body nitrogen. These data also again can be interpreted to mean that high labile protein stores protect the animal from the toxic effects of MEPA.

These studies on the dog emphasize the need to estimate the physiological state of the patient in order to prescribe safe yet adequate doses of the phosphoramides. For that reason, electrophoretic patterns have been determined in a group of animals admitted recently to the hospital. The albumin/globulin ratios of some of these patients are recorded (table 1). A normal dog with fairly full stores will have a ratio of 0.7 or higher. Many of the patients had a low normal or subnormal ratio reflecting the depleted state so often associated with cancer.

Cancer Clinic.—A cancer clinic for animals has been in operation since October, 1950. Receipt of patients is based upon prior biopsy diagnosis of cancer, admission being restricted to those types known to resist surgical and x-ray therapy, or those cases in which chemotherapy is chosen by the owner who refuses surgical or x-ray intervention for the patient.

Following admission, a few days of orientation to hospital routine and diet are permitted and then "base line" samples for blood chemistry and cell counts are withdrawn.

The patient is then assigned to a dosage schedule based upon body weight and the above clinical data. Dosage is occasionally varied slightly according to clinical judgment. Weekly blood samples are withdrawn for white cell and differential blood counts since these are used as the control data in governing drug dosage and duration of administration.

Triweekly blood samples are withdrawn for plasma electrophoresis, using the Tiselius apparatus, as well as for red blood counts and hemoglobin determinations, serology, and other blood determinations.

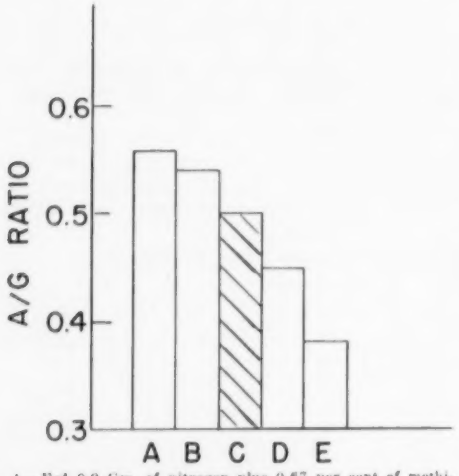
Tumor measurements, photographs, and periodic biopsies are accomplished routinely.

MEPA Therapy in Dogs.—Dosage.—The data presented here and elsewhere demonstrate that the phosphoramides can inhibit the growth of normal tissue cells,

TABLE 1—Electrophoretic Patterns of Canine Patients Admitted to the Hospital

Diagnosis	Percentage of total					
	Albumin	Globulin				A/G
		α_1	α_2	β	γ	
Control	44.0	13.0	12.0	23.0	8.0	0.79
Perianal adenocarcinoma	32.5	4.2	9.0	48.2	6.0	0.53
Perianal adenocarcinoma	22.5	12.1	13.2	40.6	11.5	0.29
Mammary carcinoma	20.1	17.2	23.1	17.2	22.4	0.25
Mammary infiltrating duct adenocarcinoma	33.0	13.7	15.9	30.2	7.3	0.49
Mammary infiltrating duct adenocarcinoma	25.7	3.9	22.6	35.0	12.8	0.35
Mammary fibrolipoma	40.8	6.6	5.8	23.4	23.4	0.69
Mammary myoepithelioma	29.4	5.6	6.7	33.6	25.4	0.39
Amelanotic melanoma	21.2	6.4	16.8	29.2	26.2	0.27
Fibrosarcoma	35.6	11.4	18.8	28.2	1.6	0.54
Fibrosarcoma	35.4	8.1	13.4	36.8	6.2	0.55
Squamous cell carcinoma	44.6	9.3	10.4	28.0	7.8	0.80
Squamous cell carcinoma	29.9	11.1	21.7	30.8	6.8	0.43
Seminoma	32.9	7.6	14.8	37.5	7.2	0.51
Transmissible venereal tumor	37.3	7.7	8.9	25.4	20.7	0.59
Malignant lymphoma	40.8	6.6	5.8	23.4	23.4	0.69
Malignant lymphoma	42.4	5.2	5.2	25.9	21.7	0.71
Malignant lymphoma	35.5	14.4	20.5	24.8	2.7	0.56
Malignant lymphoma	34.6	13.9	24.8	19.3	7.4	0.53

that they will inhibit and even cause regression of some types of tumors at dosages that have no effect upon normal tissues. The dosage for inhibition of other tumors, however, may be near, equal to, or above levels toxic to the most susceptible of normal tissue cells. If confronted with a tumor of unknown susceptibility, a high dosage near to toxic levels is often used until the action of the drug is known. It becomes important, therefore, to protect as much as possible against toxicity to normal cells. Data obtained on the dog demonstrate that MEPA (1 mg. per kilogram of body weight) given intravenously thrice weekly produced a marked leukopenia in from one to four weeks depending upon the magnitude of the protein stores and the physiological state of the animal. Daily oral administration of 5 mg. of MEPA developed a leukopenia in dogs with low protein stores such as are common in tumor-bearing animals. Further experience in normal dogs and in patients suggested that 0.7 mg. of MEPA per kilogram of body weight given intravenously thrice weekly was an effective and relatively safe dosage in patients. The intermittent administration of the drug was tried to permit maximum recovery and resistance of normal tissues to toxic effects. Leukopenia developed under these conditions but acute toxic symptoms were rarely observed. At the present time, it is believed that oral administration of MEPA is more practical and just as effective when given in doses of 2 mg. per kilogram of body weight thrice weekly. Leukopenia again may develop, but acute toxicity has not been observed so far.



A—Fed 0.2 Gm. of nitrogen plus 0.67 per cent of methionine and 0.67 per cent of glycocyamine plus 5.0 mg. of MEPA per day per kilogram of body weight.
B—Fed 0.6 Gm. of nitrogen plus 5.0 mg. of MEPA per day per kilogram of body weight.
C—Fed 0.2 Gm. of nitrogen per day per kilogram of body weight.
D—Fed 0.2 Gm. of nitrogen plus 5.0 mg. of MEPA per day per kilogram of body weight.
E—Fed 0.2 Gm. of nitrogen plus 5.0 mg. of MEPA per day per kilogram of body weight.

Graph 6—Average albumin-globulin ratios in dogs fed low casein diet.

Drug Toxicity.—Marked leukopenia and thrombocytopenia occurred prior to the development of acute clinical symptoms of toxicity. Anemia developed later, as a rule, and did not appear at all in marginal cases. By acute toxicity is meant the development of the following symptoms given in the usual order of appearance: emesis, salivation, inappetence, pyrexia (104 F. or higher), diarrhea (which may become bloody), lethargy, dehydration, dyspnea, tachycardia, limb edema, ventral edema of head, neck, and body, ecchymoses, interstitial hemorrhage, prostration, and death. Hyperesthesia associated with the severe pyrexia is occasional. Since these toxic symptoms have within them the shock syndrome, 11 of the animals showing acute toxicity were treated to overcome this condition by giving fluids and other forms of therapy such as doses of antihistamine calculated under label directions. Although the number of animals was small, the results demonstrate that this therapy was inadequate, the animals dying in shock. Later, given much larger doses of antihistamine, 12 of 17 animals showing severe toxic symptoms recovered. During recovery from toxicity, the body temperature returned to normal in 12 to 72 hours, appetite returned and there was restoration of blood cell counts in from five days to six weeks depending upon the severity of the toxic reaction and the animal's reserve stores. The corrective therapy for acute toxicity, at present, is antihistamine in a dosage 50 per cent over the maximum calculated dose as directed, given one to three times daily in Ringer's solution, the latter totalling 600 to 1,000 cc., subcutaneously. Antibiotics are administered for the prevention of secondary

TABLE 2—Summary of Case Roster of Dogs Bearing Spontaneous Cancer Treated with MEPA,* Showing Diagnosis, Unit Dosage, Means of Administration, Total Dose Given, and Therapeutic Response

Case (No.)	Diagnosis	MEPA (mg./kg.)	Route	Total dose (mg.)	Therapeutic response
CR 30	Fibrosarcoma, gingival	0.2 0.5 1.0 2.0	i.v. i.v. i.v. oral	2033.4	Entered clinic 3/3/51. Received 64.66 mg. TEM** total dose, electromagnetic wave therapy prior to MEPA. Possible cervical lymph node metastasis evident after TEM therapy. Growth retarded. Still under treatment.
CR 69	Infiltrating papillary duct adenocarcinoma, mammary gland	0.09 0.10	i.v. i.v.	18.5	Surgical recurrence, scar line secondaries. Degeneration, encapsulation, partial regression. Telemetastases. Death in four months.
CR 85	Fibrosarcoma, nasal subcutis	0.09 0.15 0.50	i.v. i.v. i.v.	98.85	Retarded infiltration at base of tumor, but not growth in pedunculated portion. No metastases found at necropsy. Death in three months.
CR 90	Myoepithelioma, mammary gland	0.70	i.v.	95.2	Encapsulation, pendulation, extirpation. On follow-up study.
CR 92	Squamous cell carcinoma, anterior nares	1.0	i.v.	255.6	Retarded infiltration and growth. No metastases found at necropsy. Death in two months.
CR 93	Malignant lymphoma	1.0	i.v.	42.0	No regression. Death in five days. Advanced case.
CR 94	Malignant lymphoma	1.5	i.v.	240.0	One remission of two weeks followed by second regression and death. Toxic dose.
CR 97	Myoepithelioma, mammary gland	1.0	i.v.	120.0	No effect. Death in two months.
CR 98	Infiltrating papillary duct adenocarcinoma, mammary gland	1.0	i.v.	61.2	Multiple pulmonary metastases at entry. Death in six days from start of therapy.
CR 99	Malignant lymphoma	1.0 5.0	i.v. oral	367.0	First remission lasted 3½ months after complete regression. Second regression was followed by death after two weeks' remission. Death in eight months.
CR 100	Fibrosarcoma, gingival	1.0	i.v.	263.2	Retarded infiltration. Inhibited metastasis. Studied as aged patient with chronic lack of appetite and irregular food consumption record. Death in one month.
CR 101	Malignant lymphoma	1.0	i.v.	51.86	First remission lasted two weeks, second lasted one week. Died one week after second recurrence.
CR 102	Infiltrating papillary duct adenocarcinoma, mammary gland	1.0	i.v.	77.8	Highly malignant growth visible daily. Involved entire mammary line in three weeks from onset. Some degeneration in glandular fraction but no apparent effect on connective tissue. Death in two weeks.
CR 103	Fibrosarcoma, dorsal thoracic soft tissue	1.0 2.0 3.0 2.0	i.v. oral oral oral	906.0	Patient in ninth month of therapy. Retardation of tumor growth. Tumor now movable, formerly fast to vertebra. Considered surgically approachable.
CR 104	Malignant lymphoma	1.0	i.v.	205.0	Complete regression but died five days later at referring hospital.
CR 105	Seminoma	2.0	oral	3600.0	Slight reduction in size of scrotal mass and increasing firmness. Still under treatment.
CR 106	Perianal adenocarcinoma	1.0	i.v.	177.0	Regression almost complete when death intervened. Limited food intake favored toxicity. Death in three weeks.
CR 108	Infiltrating neoplasm of penis, undiagnosed	2.0	oral	445.0	Inhibition of infiltration, fibrotic change and encapsulation. Now a surgical problem. Not biopsied due to possibility of atresia of urethra, will be classified after surgical removal.
CR 109	Squamous cell carcinoma	2.0	oral	160.0	Degeneration, with increased differentiation compared to biopsy specimen of tumor. Euthanized for histopathology material.
CR 111	Infiltrating papillary duct adenocarcinoma, mammary gland	5.0	oral	700.0	Postsurgical carcinomatosis prevailed at entry. Under therapy, degeneration of tumor tissue in axillary and pulmonary metastases. Euthanized for histopathology material.
CR 112	Malignant lymphoma	5.0	oral	200.0	Lymph node regression 80% at death after three weeks of therapy.
CR 114	Mast cell sarcoma	3.0 2.0	oral	697.0	Cutaneous sarcomatosis; 30% of cutaneous metastases regressed. Death in two months.
CR 115	Infiltrating papillary duct adenocarcinoma	2.0	oral	315.0	Degeneration of ulcerating "buds" of tumor, encapsulation, pendulation, extirpation. Under observation.
CR 116	Spindle cell sarcoma hard palate, gingiva	3.0	oral	480.0	Change in tumor color from deep red to whitish pink. Still under treatment.
CR 117	Malignant lymphoma	2.0	oral	60.0	Regression of lymph nodes 80%. Under treatment.

* N-(3-oxapentamethylene)-N', N''-diethylenephosphoramide; ** 2,4,6-triethylenimino-s-triazine; i.v.—intravenous.

bacterial infections attributable to lowered resistance during the concurrent leukopenia.

Tumor Therapy.—A record of animals that have received MEPA, the diagnosis, dose, and response is shown (table 2). As reported previously, the phosphoramides are most effective in producing regression of malignant lymphomas. Malignant lymphoma is a uniformly fatal disease in the dog characterized by a generalized hyperplasia of the lymphopoietic cells and consequent lymph node and splenic enlargement. There may be an increase in types of mature and immature granulocytes in the circulation. The disease is usually fatal within a few weeks to a maximum of seven months after the diagnosis.[14,15] Seven cases of malignant lymphoma have been treated with MEPA. The majority were in an advanced stage of the disease when it was diagnosed. MEPA produced regressions of lymph nodes in 6 of the 7 dogs. With the doses used, 3 had one regression; 3 had a regression, followed by a remission and then another regression; and 1 has had three regressions with remissions covering eight months. The rapid regressions of the lymph nodes in the presence of MEPA were always accompanied by toxic symptoms similar to, but not thought to be, the result of drug toxicity alone. This toxicity, however, was treated successfully by using fluids and large doses of the antihistamine. Regressions were obtained, possibly lives were lengthened but, if this form of therapy is to be successful, a dose must be found which will bring about a more gradual regression with less toxicity to the patient. The present oral dosage of 2 mg. per kilogram of body weight thrice weekly is being used for that purpose.

Histologically, the regressed lymph node is somewhat restored to its original architecture in that pericapsular infiltrations of lymphocytes or lymphoblasts, depending on the type of disease present, are no longer in evidence, and the peripheral sinus is restored. The germinal centers, vascular spaces, and medulla of the gland are not restored, however, for in the regressed nodes the picture is one of a homogeneous sheet of lymphopoietic cells which retain the potential for recurrence.

Seven dogs with mammary cancer have been treated with MEPA. Of these, five cases have been infiltrating papillary duct adenocarcinoma and two have been malignant myoepithelioma. One of the five infiltrating duct adenocarcinomas has been extirpated after obvious tumor degeneration, partial regression, and pendulation. This change had been shown also by two earlier patients at necropsy, wherein the therapy had been continuous in hope of possible regression but had been too prolonged and had caused their death. Two others of the 5 patients had multiple pulmonary metastatic tumors. The first received intravenously 1 mg. of MEPA per kilogram thrice weekly and died evidencing toxic symptoms five days following the first series. The second received 5 mg. of MEPA per kilogram orally twice weekly, eventually developing shock symptoms and dying, however. The response of multiple pulmonary metastases to MEPA was not good. Terminal pulmonary edema and an occasional hemorrhage developed, possibly because of an effect upon the widespread neoplastic tissue in the lungs. One of the two malignant myoepitheliomas responded to MEPA therapy with retarded growth and pendulation and was extirpated successfully. The other case showed no response to the drug.

A total of 100 cases has been studied to date. Statistically, they present the following data:

1) types, malignant lymphoma, 37; mammary cancer, infiltrating duct, 9, infiltrating lobular, 3; squamous cell carcinoma, 9; spindle cell sarcoma, 4; merocrine adenocarcinoma, 4; lymphoblastoma, 2; mast cell sarcoma, 3; leiomyosarcoma, 2; myxofibrosarcoma, 2; osteogenic sarcoma, 2; apocrine adenocarcinoma, 2; neurofibrosarcoma, 1; seminoma, 2; amelanotic melanoma, 3; periosteal fibrosarcoma, 1; basal cell carcinoma, 1; histiocytoma, 1; fibrosarcoma, 8; fibrolipoma, 1; malignant myoepithelioma, 2; unclassified penile tumor, 1;

2) incidence by sex, male patients, 53; female patients, 47;

3) incidence by age, 1-4 years, 20; 5-8 years, 30; 9-12 years, 45; 13-16 years, 5.

This list includes those animals described in table 2 as having been treated with MEPA.

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Three Methods of Inducing Graded Obstruction of the Pulmonary Circulation

CAPTAIN JOHNIE L. REEVES, V.C., U.S.A.F.; JAMES P. HENRY, M.D.; OTTO H. GAUER, M.D.

Dayton, Ohio

Literature Cited

Several studies have demonstrated the effects of changes in blood volume on urine flow.[1-6] In order to determine the factors responsible, it was necessary to develop means of changing the volume of individual segments of the vascular tree. Farber, Beeker, and Eichna[4] have used a balloon to distend either the inferior or the superior vena cava and Fishman[7] has distended the vessels in the head. The negative or absent effects of these procedures on urine flow contrasts with the diuresis induced by negative pressure breathing,[8,9] or by changes in posture which increase the volume of blood in the chest[10] and points to the thoracic vascular bed as a possible site of receptors responsible for urine changes. The present report presents three methods of inducing graded obstruction of different portions of the pulmonary circulation. They have been found useful in localizing receptors believed to link blood volume changes with alterations in kidney function. Briefly stated, the techniques used were plastic bead blocking of radicles of the pulmonary artery, snare closure of the pulmonary veins, and balloon inflation in the left auricle. The experimental results are reported elsewhere.[11]

Materials and Methods

Young, mature, mongrel bitches weighing between 9 and 18 kg. were used. All animals were given 15 mg. of morphine sulphate as a preanesthetic sedative. Anesthesia was induced by slow intravenous injection of 8 to 10 cc. per kilogram of body weight of a warm solution containing 1.0 per cent of chloralose * in 0.6 per cent of sodium chloride. The chloralose solution was given until the animal's pupils contracted and the eyes rolled forward hiding the greater part of the iris. At this point, the animal was surgically anesthetized, yet still exhibited a pronounced reflex jerk when the table on which she lay was tapped. Usually, no additional anesthetic was needed during the surgery. However, if the operative procedure exceeded an hour, supplementary anesthetic was occasionally given. Postoperatively, as in the earlier work by Gauer *et al.*,[6] the anesthesia was allowed to lighten to the highest plane compatible with quietude and was maintained at that level by intravenous injection of approximately 5 mg. per kilogram of chloralose every ten minutes. Hydration was maintained by adding enough physiological saline to the chloralose solution to make a total injection of 1 cc. per kilogram of body weight every ten minutes.

It was found that the chloralose solution could be kept in a water bath at 65 to 75 C. for 12 hours or more without causing a disturbance of urine flow, or other toxic symptoms. Accordingly, the solution was made up in 240-cc. batches, enough for six hours or more, and was kept warm in an Erlenmeyer flask in a water bath. Evaporation was eliminated by closing the orifice with a stopper having a tube running through it to the bottom of the flask for drawing the solution.

All surgery from which the animal was to recover was done using sterile technique. However, the bulk of these studies has been acute, and the surgical preparation has consisted of clipping and scrubbing the area to be operated and draping with a suitable laparotomy sheet in an effort to keep gross hair and dirt contamination to a minimum. A routine precautionary dose of 300,000 units of penicillin ** was given intramuscularly the day before to inhibit possible overwhelming gram-positive bacterial invasion during the 10- to 14-hour period of postsurgical experimental procedure.

Recordings were routinely made of pulmonary arterial, central venous, and systemic arterial pressures and of ten-minute urine volumes. For recording pulmonary arterial pressures, a No. 8 cardiac catheter was introduced by way of the jugular vein and right heart chambers until the tip was in the pulmonary artery and just on the edge of the heart shadow as visualized under the fluoroscope. Central venous pressures were taken by inserting a 2-mm., inside diameter, vinylite tube by way of the femoral vein into the posterior vena cava to the level of the fifth or sixth rib near the base of the heart. Systemic arterial pressures were recorded by means of the same size vinylite tubing introduced by way of the femoral artery into the descending aorta. For the balloon and some of the snare experiments a 1-mm., inside diameter, vinylite tube was introduced into either the left atrium or a pulmonary

From the Aero Medical Laboratory, Wright Air Development Center, Wright-Patterson Air Force Base, Ohio.

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* Alpha-chloralose, Bios Laboratories, Inc., 17 W. 60th St., New York 23, N. Y.

** Dibenzylethylenediamine dipenicillin G "Bicillin L-A," Wyeth Inc., Philadelphia, Pa.

vein, or both, at the time of the thoracotomy for recording pressures in these structures.

Anticoagulants were used sparingly in all experiments, to keep blood loss to a minimum. The usual procedure was to measure the volume of each catheter and, when recordings indicated damping due to clotting, the anticoagulant in the catheter was drawn into a 30-cc. syringe full of saline and a small amount flushed back through. A second syringe containing 35 mg. of sodium citrate and 100 U.S.P. units of heparin per cubic centimeter of saline was then used to fill the catheter to its tip. The urinary catheter was made of 6-mm., outside diameter, glass tubing curved to fit the bitch's urethra. The tip was probe pointed and three rows of holes perforated the proximal 6 cm. A latex tube led from the distal end of the glass catheter to a burette placed on the floor. This 50- to 60-cm. column of urine exerted enough suction to keep the bladder empty.

Plastic Beads

For the plastic bead† injection, the only surgery necessary was to make small incisions over the jugular vein and the femoral artery and vein to receive the vascular catheters. The beads were prepared for infusion by repeated washing in distilled water and centrifuging. When the urine flow was stable, a 10 per cent by weight suspension of these beads in physiological saline was infused in 2-cc. aliquots at five-minute intervals through a double-lumen pulmonary artery catheter. Approximately 1 cc. per kilogram of body weight was usually needed to increase pulmonary arterial pressure by 15 to 20 cm. of water. The first few infusions had no effect, but as the arterial tree filled the infusions caused a marked rise in pulmonary artery pressure. After five or ten minutes, this pressure would fall off to 30 to 50 per cent of its peak immediately following the infusion. A further infusion of 1 to 2 cc. was necessary to restore the hypertension to the desired level. At this stage, the dosage was critical and a slight excess of beads would lead to an intense pulmonary hypertension and to a fatal fall in systemic arterial pressure. The fall of pulmonary artery pressure could have been due to dilatation of the capillaries allowing the passage of a portion of the beads, or to opening of pulmonary arteriovenous shunts.[12,13] That at least a portion of the beads was not arrested by the pulmonary capillaries was proved by finding them in microscopic sections of the brain and kidney at necropsy.

Pulmonary Vein Snares

These snares were made after the style of those devised for mitral stenosis by Ellison *et al.*[14] One end of a strand of flexible stainless steel cable,‡ approximately 50 cm. long, was threaded into a blunted and curved 19-gauge hypodermic needle shaft and the shaft crimped firmly about it. A

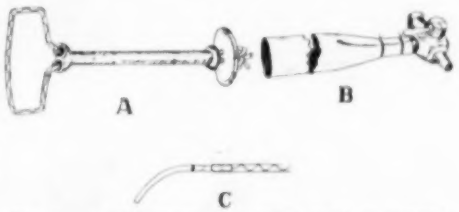

Fig. 1—A pulmonary vein snare used to congest circulation showing (A) vinylite tube shaft with snare in place and "boxed" as it would be when surrounding the pulmonary veins; (B) penrose tubing which is slipped over the button and tied securely to the shaft; (C) snare wire assembly showing the hypodermic needle probe crimped to a strand of cable and the vinylite sheath used to protect the veins from the cutting action of the cable.

vinylite tubing sheath was then slipped over the wire and a portion of the needle. The snare shaft was made of a stiff 4-mm., inside diameter, vinylite tube approximately 10 cm. long. The ends of this tube were heated until they flared into a funnel of approximately 1 cm. in diameter. One end was formed into a Y by compressing opposite sides of the funnel and the other end was flattened to form a plate. Several small holes were melted into the end near the Y to provide for postoperative drainage of fluids from the thorax (fig. 1). Placement of these snares required a bilateral thoracotomy. No preference was given to which side was operated first.

An endotracheal tube with an inflatable cuff and a modified Seeler resuscitator was used to provide artificial respiration.[15] The rate and depth of respiration were adjusted to duplicate those immediately preceding attachment of the resuscitator. The pressures were set at not less than minus 1 mm. of mercury on the expiratory cycle and not more than plus 10 mm. of mercury on the inspiratory cycle. The approach to the lung hilus on either side was made by resecting a portion of the fifth rib. Much muscular twitching was encountered, so it was wise to mark the line of the skin incision with a scalpel tip before making the cut. Somewhat more hemorrhage is to be encountered under chloralose anesthesia than with less voluminous anesthetic agents. Therefore, blunt dissection, careful control of even minor bleeding, and sparing use of anticoagulants was essential in preventing postoperative decrease in blood volume. The thorax was opened and the lung carefully reflected dorsally with the finger or a saline moistened sponge as the probe point of the snare wire was threaded under the pulmonary veins as close

† Dow Chemical Company, Midland, Mich., experimental plastic beads, X-772, 200–400 mesh 40–80 μ diameter, formulation P-9-5-4.

‡ American Chain Cable, Inc., 3 x 7 x .032 outside diameter of cable.

to the pericardium as was possible. The loose ends of the wire were then passed through the arms of the Y and up the snare shaft so that a loose loop was formed. In most cases, it was necessary to crimp the loop to make a "box" around the veins to prevent compression of those from the apical lobe and, in the case of the right lung, also those from the intermediate lobe. The Y guided the limbs of the loop away from each other and insured that the loop would be reformed fully after it had been closed sufficiently to occlude the veins. A large button was then slipped over the loose ends of the wires and down to the flattened end of the shaft and a large knot was tied in the snare

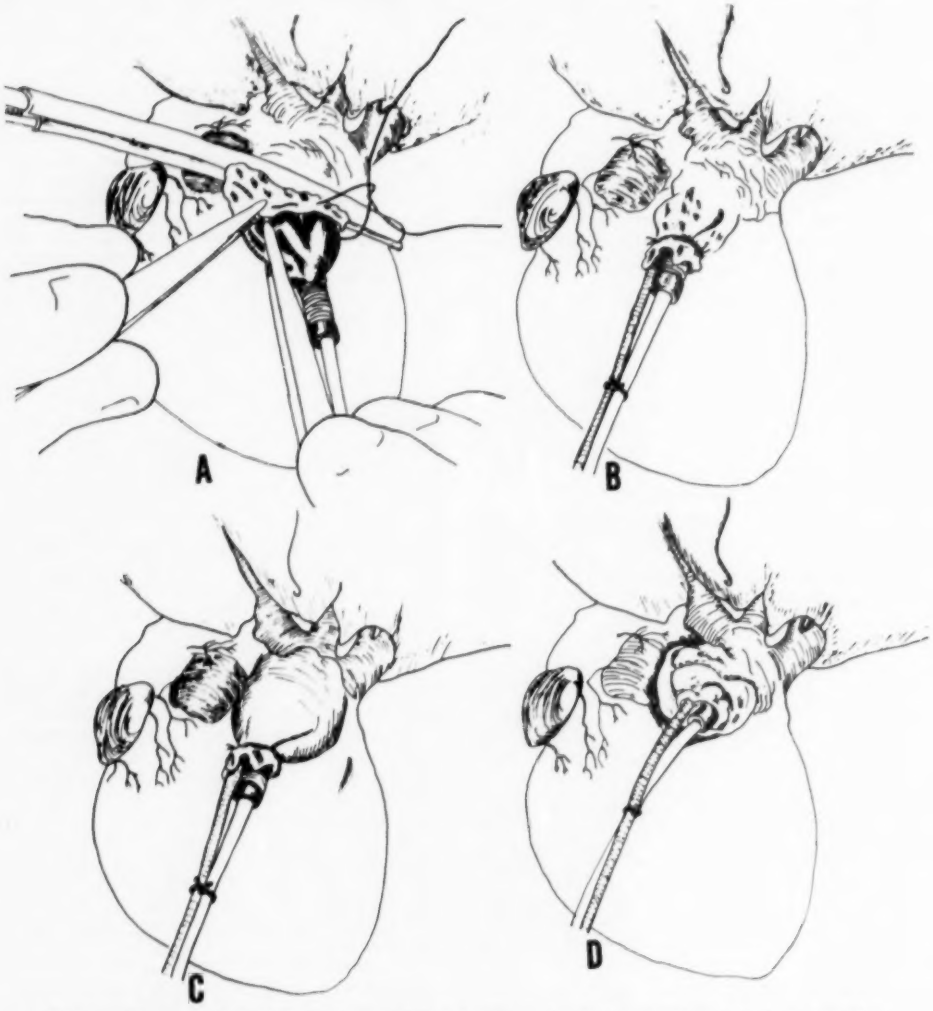

Fig. 2—The use of the balloon to congest pulmonary circulation showing (A) inserting the balloon assembly into the auricle; (B) balloon in place and uninflated; (C) balloon inflated and tension placed on the tubes to prevent invagination (this can be done in the open but not satisfactorily in the closed chest), auricle noticeably stretched; (D) balloon inflated and tension released to allow the balloon to move into the atrium and block the mitral orifice. The auricle invaginates and, at approximately 15 cc. of inflation, allows the balloon to protrude through the mitral ring into the ventricle with each auricular systole.

wires. An 8-inch length of silicone-coated 1-inch penrose drainage tubing was then slipped over the flattened end of the snare shaft and tied securely. To the other end of the penrose tubing was fixed a three-way stopcock.

The lung was then carefully returned to its original position and the thorax was closed using at least two layers of sutures in an effort to make the closure absolutely airtight. The contralateral side was then operated and a similar snare placement made. In most cases, inclosure of the vein from the intermediate lobe of the right lung could not be achieved from the right side; however it, could be reached from the left side and included in the snare surrounding the left veins. It was necessary to puncture the venacaval fold of pleura to allow the snare to pass around the vein of the intermediate lobe if it were approached from the left side. Frequently the vein from the right diaphragmatic lobe or some small accessory vein failed to be inclosed within the snares, but usually enough of the lung circulation could be obstructed to induce the necessary 20 to 25 cm. of water increase of mean pulmonary arterial pressure.[6, 11] At the completion of the surgery, the pneumothorax and hemothorax that may have occurred was reduced by drawing the foreign substances through the stopcock in the penrose tubing.

The usual experimental procedure with the snares was to pull one snare loop snug and clamp it near the button, then to tighten gradually the contralateral snare until the desired increase in pulmonary artery pressures occurred.

Left Auricular Balloon

The rubber balloons used to distend the auricle and to obstruct the blood flow from the pulmonary veins were molded on a 2-cm. diameter glass sphere with a 5-mm., outside diameter, stem. They were attached to a 2-mm., inside diameter, soft vinylite tube, the tip of which had been built up to 5 mm., outside diameter, to fit snugly within the balloon stem. A 2-mm., inside diameter, vinylite pressure-recording catheter was tied alongside the balloon and its inflating tube, and the whole was coated with silicone jelly. As in the snare placement, thoracotomy was done at the fifth rib but in this case only the left side was invaded. The lobes of the left lung were carefully separated at the cleft between the cardiac and diaphragmatic lobes so that the pericardium could be opened and drawn toward the incision to form a cradle in which the heart lay during the manipulations of the left auricular appendage. A clamp was placed obliquely across the appendage about 1 cm. from the tip and posterior border. Care was taken to clamp just tight enough to prevent leakage of blood. A purse string suture was placed around the tip of the auricle near the clamp, a stab wound made through the margin inclosed by the suture, and the balloon assembly introduced into the left auricle through the wound. The insertion of the balloon can be made with very little loss of blood if one assistant will hold the margins of the wound open with two mosquito forceps as the operator inserts the first part of the balloon assembly and if a second assistant releases the auricular clamp as the operator completes the insertion and tightens the purse string. The chest was closed as described above. The pneumothorax was reduced and spontaneous respiration was re-established.

It was found that if the auricular pressure catheter tip extended slightly beyond the edge of the collapsed balloon and if it were directed upstream, that is, toward the pulmonary veins, less interference with the tracing due to blocking by the heart wall would occur. The balloon was placed so that when it was collapsed it rested mainly in the appendage. When it was inflated during an open chest observation, it would move into the blood stream and invaginate the auricular appendage to a varying degree (fig. 2). Cinefluorography confirmed an earlier suspicion that the balloon on occasion protruded through the mitral valve into the ventricle at atrial systole and returned to the atrium at ventricular systole. The experimental procedure involved attaching the balloon catheter to a 30-cc. saline-filled syringe and filling the balloon to the desired degree as indicated by the pressures recorded on the oscillograph.

Summary

Description of the techniques and apparatus used in 62 experiments for producing graded obstruction of the pulmonary circulation is presented. They proved of value in determining the precise locus of receptors which induce changes in kidney function in response to alterations in blood volume. Specifically, infusion of plastic beads was used to congest the pulmonary circulation upstream from the small pulmonary artery radicles, snares to congest upstream from the retropericardial pulmonary veins, and a balloon to congest upstream from the left atrium (mitral valve).

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Encephalitis in Dogs Produced by Distemper Virus

JAMES H. GILLESPIE, V.M.D. and CHARLES G. RICKARD, D.V.M., M.S.

Ithaca, New York

ENCEPHALITIS in dogs manifested by epileptiform convulsions was associated with distemper in earlier studies on this disease.[1] Recently, however, there has been some question whether distemper virus as such could cause encephalitis.[2] By some, the cause has been attributed to a virus other than distemper virus[2] and by others, either to a mutant of distemper virus or else caused by distemper virus aided by hypothetical toxins.[3] In any case, there seems general agreement that the basic factors influencing distemper virus to produce encephalitis are poorly understood.

Major difficulty in the study of this problem has been the failure to produce nervous manifestations with regularity. In this work on encephalitis, therefore, an agent and a method were sought that would produce neurological signs regularly. Results of this search form the basis of this report.

ISOLATIONS OF STRAIN USED FOR STUDY

In September 1950, a dog without neurological complications that showed only generalized signs of distemper was presented for study. After euthanasia, a necropsy was performed; portions of cerebrum and cerebellum were removed, pooled, and placed in vials under dry ice refrigeration. Other portions of brain were saved for histological and bacteriological studies.

In a test for virus, a vial of cerebrum and cerebellum was thawed and a 10 per cent suspension made in saline with a glass grinder to be used as inoculum. Prior to inoculation, each dog was anesthetized with sodium pentobarbital. Then the head was shaved, painted with tincture of iodine, a median incision 5 mm. in length was made, the skin was deflected laterally, and a hole was drilled through the skull. Care was taken that the drill did not cause destruction of underlying tissue. Using a 1-ml. syringe with a 26-gauge needle, ¾ in. in length, 0.3 ml. of the brain suspension was inoculated intracerebrally into each dog. The syringe was held at a 45 degree angle so that the inoculum was introduced into a cerebral hemisphere. After inoculation, the skin was returned to normal position, and covered with a piece of sterile gauze and cotton. The bandage was held in place by a large overlying piece of gauze which was fixed to the skin by the use of surgical adhesive. Usually, the bandage stayed in position until the skin incision had healed.

In the first transfer, 2 dogs were inoculated and each showed an elevated temperature six days after inoculation. One of these was destroyed and its brain removed for further serial transfer. The other dog, held under observation, showed the usual course of distemper without neurological signs and died with pneumonia 32 days after inoculation. In the second serial passage, 2 dogs again were inoculated. Both dogs developed elevated temperatures and brain specimens were obtained from 1 two days later. Neurological signs were seen in the other animal 29 days after inoculation. This dog was destroyed two days later and its brain used for a third serial passage into 2 other dogs. The brain was removed from 1 dog, after an elevated temperature had developed, and was used for a fourth serial passage. Both dogs showed elevated temperature and 1 was destroyed two days later. The other animal developed neurological signs 22 days after inoculation, was destroyed one day later and its brain was used for the fifth serial passage. In subsequent serial passages, clinical evidence of brain involvement has appeared in all dogs except 1 six to 16 days after inoculation.

It appeared that an infectious agent, designated Snyder Hill, had been procured that was capable of producing nervous manifestations regularly in dogs inoculated intracerebrally. Brain material from various passages when cultured on blood agar slants and in sodium thioglycolate medium, showed no growth of microorganisms, and microscopically revealed no visible bacteria. Histological examination of brain and other organs showed inclusion bodies typical of those associated with distemper. Accordingly, distemper virus was obtained from several sources and compared in cross-immunity tests with the Snyder Hill agent.

In the cross-immunity tests, four strains of distemper virus were used: (1) a strain obtained from the Fromm Laboratories,* (2) another obtained from a dog showing hardpads and neurological involvement** called Buffalo strain, (3) an egg-adapted strain called Onderstepoort strain,† (4) and another egg-adapted strain obtained from Lederle Laboratories.‡ In a test, a dog was inocu-

From the Veterinary Virus Research Institute (Gillespie) and the Department of Pathology and Bacteriology (Rickard), New York State Veterinary College at Cornell University, Ithaca.

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* Through the courtesy of Dr. Harold Siedentopf.

** Furnished by Dr. Robert McClelland of Buffalo, N. Y.

† Furnished by Dr. Haig of South Africa.

‡ Through the courtesy of Dr. Victor Cabasso.

lated with virus intravenously and observed for signs of illness for 30 days. After illness and the development of neutralizing antibodies, it was reinoculated with the same strain, and after another 14 days inoculated with the other strain. After each inoculation, the dog was observed for 14 days for signs of illness, temperature elevations, and leukocyte variations. Failure to show signs of illness following reinoculation indicated cross immunization. These results are presented (table 1) where it can be seen that each strain immunized against itself and against the Snyder Hill agent. In addition, serums from dogs infected with Fromm, Lederle, Onderstepoort and Snyder Hill strains were collected before inoculation and again after recovery. Each serum was tested for neutralizing capacity in eggs against either the Onderstepoort or the Lederle strain of egg-adapted virus. In this test, between 100 to 1,000 infectious doses of virus were used for each egg. Twofold dilutions of a serum were prepared and each dilution mixed with virus, placed in a refrigerator for two hours and 0.2 ml. of each mixture then inoculated on the chorioallantoic membrane of five eggs that had been incubated for seven to eight days. After further incubation for seven days, the chorioallantoic membrane was examined for lesions. Failure to find lesions indicated neutralization. Before inoculation, the serums from none of the dogs neutralized virus in a dilution of 1:5. After infection, and recovery, all serums neutralized virus in dilutions that ranged from 1:160 to 1:1280. Since serums from dogs which had recovered from infection with the Snyder Hill agent neutralized virus the same as did serums from dogs that had recovered from known strains of distemper, this was considered further proof of the antigenic similarity of the Snyder Hill agent to distemper virus. It was concluded, therefore, that the Snyder Hill agent was distemper virus.

TABLE 1—Cross-Immunity Tests in Dogs of the Snyder Hill Strain with Four Other Strains of Distemper Virus

DOGS IMMUNIZED WITH THE SNYDER HILL STRAIN	
Test Strain	
Fromm	0/5
Buffalo	0/3
Lederle	0/5
Onderstepoort	0/3
Snyder Hill	0/20*
DOGS CHALLENGED BY THE SNYDER HILL STRAIN	
Immunizing Strain	
Fromm	0/5
Buffalo	0/2
Lederle	0/5
Onderstepoort	0/3
Snyder Hill	0/20*

* Same dogs.

Numerator—number of dogs affected; denominator—number of dogs inoculated.

When the dogs were immunized for this comparison, those inoculated with the Fromm, Buffalo, and Snyder Hill strains, all showed signs of illness. Those inoculated with the Onderstepoort or with the Lederle strains had mild or no clinical signs.

In all immunized dogs, antibodies were demonstrated which neutralized the Onderstepoort or the Lederle strain in embryonated eggs.

DISEASE IN DOGS PRODUCED BY SNYDER HILL STRAIN OF DISTEMPER VIRUS

Production.—The dogs used in the study were mongrels. They were 12 to 20 weeks of age, except one litter of 5 which were 5 weeks old, and all had been procured from farms near Ithaca. Litters of 4 or more dogs were obtained, placed in isolation, and observed for a week or more for signs of illness. One dog from each litter was tested for susceptibility to distemper virus before the others were used. When they developed illness following inoculation their litter mates were considered susceptible to distemper and suitable for this study.

TABLE 2—Neurological Manifestations Shown by Dogs Inoculated with the Snyder Hill Strain of Distemper Virus

Route of inoculation	Epileptiform convulsions	Myoclonus	Death without observed neurological signs	Total
Intracerebral	51/60	1/60	5/60	57/60
Intravenous	8/32	6/32	0/32	14/32
Subcutaneous	3/12	2/12	0/12	5/12
Intranasal	0/4	0/4	0/4	0/4
Contact	4/11	0/11	0/11	4/11

Dogs were observed for longer than 45 days.

Numerator—number of dogs affected; denominator—number of dogs inoculated.

Dogs were inoculated intracerebrally and also by other methods for comparison. For inoculations intracerebrally, continuous serial passages were made as previously described using 1 or 2 dogs for each transfer. In all, 66 dogs were inoculated in 35 serial passages. For other routes of inoculation, 1 ml. of a 10 per cent suspension in saline of brain or of spleen with virus that had been transferred nine or more passages, intracerebrally, was used. In these tests, 32 dogs were inoculated intravenously, 12 subcutaneously, and 4 intranasally. In addition, 11 dogs were exposed by placing them in the same room with others that had been infected.

Following inoculation, all dogs were observed daily for 45 days or longer unless convulsions or death supervened. Temperatures were taken each day and leukocyte counts were made daily on most of the dogs. Prior to inoculation and again two to four weeks afterward, blood was obtained from some dogs and serum separated from the clot for neutralization tests. Also, 31 of the dogs inoculated intracerebrally were destroyed after the development of neurological manifestations and portions of brain were removed from each for pathological study.

Results.—Of 60 dogs inoculated intracerebrally (table 2) and observed for 45 days or longer, 51 showed epileptiform

convulsions, 1 developed myoclonus, 5 died without observed neurological signs, and 3 showed generalized signs of illness only. Of 32 dogs inoculated intravenously, 8 showed epileptiform convulsions while 6 developed myoclonus and recovered. Of 11 dogs exposed by contact, 4 showed epileptiform convulsions, and of 12 inoculated subcutaneously, 3 showed epileptiform convulsions and 2, myoclonus. Of 4 inoculated intranasally, none showed neurological signs.

Clinical Features.—Dogs given virus intravenously usually developed an elevated temperature two days after inoculation although in some the onset was delayed as long as eight days. Similar incubation periods were found for dogs inoculated intracerebrally although an occasional dog failed to show a temperature elevation until the onset of nervous manifestations. Elevated temperatures developed in about five days in dogs inoculated subcutaneously and in about six days in those inoculated intranasally or exposed by contact. Usually the temperatures followed a diphasic pattern although some remained high for most of the illness. Leukopenia was found in most dogs and the leukocyte count remained low throughout the illness. The usual catarrhal signs of illness, such as congestion of the conjunctival membrane and a serous discharge from the eyes and the nose, developed shortly after the onset of fever. Later, diarrhea usually developed and a few dogs showed signs of pneumonia. Except for a few showing myoclonus, all dogs that developed marked neurological complications died. All others recovered except a few that developed pneumonia.

Most of the dogs with nervous manifestations had a syndrome characterized by several or all of the following: depression, myalgia, myoclonus, incoordination, circling, epileptiform convulsions, coma, and death. The epileptiform convulsions usually occurred one to two days before death.

Pathology.—Brains from 31 dogs that showed neurological manifestations furnished material for this study. In addition, 2 uninoculated dogs and 3 that were inoculated intracerebrally with inactivated virus were included for comparison. Inactivation of the Snyder Hill strain in brain tissue was attained by heating the 20 per cent suspension at 60 C. for three hours. After necropsy, brains were removed and 22 of them placed in a large volume of 10 per cent aqueous formalin solution for about one week at room temperature. After fixation, thin slices of tissue were cut from various parts of the brains. In the preparation of brains from 3 dogs in which convulsions had occurred and from 2 uninoculated dogs, thin slices were cut, placed in susa fixative and in 10 per cent formalin, and kept in the refrigerator for 24 hours. The brains from the other dogs which had shown nervous manifestations, and from the 3 which had been given inactivated virus, were placed in a large volume of 10 per cent aqueous formalin immediately after euthanasia and refrigerated for one week; then, thin slices were cut. After fixation, slides were prepared, stained with hematoxylin and eosin and, in some cases, with azure eosinate and Mallory's phosphotungstic acid hematoxylin. Also, frozen sections of formalin-fixed cerebellum from some infected dogs and from 5 control dogs were chromated for three days and stained by Lillie's technique for myelin and degenerated myelin.

The cerebellums of all the 31 dogs that developed nervous manifestations showed perivascular cuffing, nonsuppurative lepto-

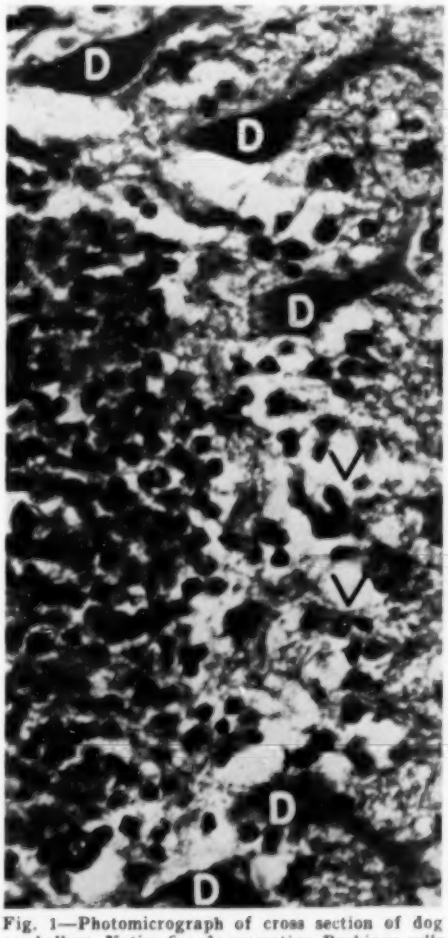

Fig. 1—Photomicrograph of cross section of dog cerebellum. Notice five degenerating Purkinge cells (D) and vacuoles (V) where two others apparently have disappeared. Hematoxylin and eosin. x 760.

meningitis with variable accumulations of mononuclear cells, neuronal degeneration, and vacuoles in the white matter. Many Purkinje cells showed degenerative changes (fig. 1). Numerous cells showed pyknosis while other cells appeared swollen and their Nissl granules were small and indistinct. Some Purkinje cells had a zone free of Nissl granules around the nucleus and the nuclei of some cells had migrated to the edge of the cell. Other Purkinje cells had faded so that they were almost unrecognizable and apparently were undergoing cytolysis. Neuronophagia was not recognized. Vacuoles were seen in the white matter of the cerebellum of inoculated dogs; however, some similar small vacuoles were found in the control dogs. The vacuoles did not have a perivascular distribution. Degenerated myelin was not demonstrated in frozen sections of cerebellum from dogs that were destroyed seven to 16 days after inoculation. Gliosis was seen in the cerebellums of a few dogs and, in general, was most marked in dogs that developed nervous manifestations 22 or more days after inoculation or contact with infected dogs. Uninoculated dogs and dogs inoculated with heat-inactivated Snyder Hill virus did not show any of these changes.

Fig. 2—Photomicrograph of cross section of dog cerebellum. Notice glial cells with intranuclear inclusion bodies (arrows). Hematoxylin and eosin. x 760.

TABLE 3—Effect of Intracerebral Inoculation of Snyder Hill Strain of Dogs Recovered from Virulent and from Attenuated Egg-Propagated Distemper Virus

Immunizing strain	Results of intracerebral inoculation of adapted Snyder Hill strain
Snyder Hill	0/20
Egg-propagated virus	0/5
Uninoculated	57/58

Numerator—number of dogs that showed neurological manifestations or death; denominator—number of dogs tested.

Numerous gitter cells and intranuclear inclusion bodies in glial cells (fig. 2) were found in the cerebellums of the 2 dogs that developed neurological disturbance after the longer intervals, 22 and 29 days. Tissues suitable for frozen sections and myelin stains were not available from these dogs, but the gitter cells indicated a demyelinating process (fig. 3 and 4). Inclusion bodies were not found in the cerebellums of the dogs that developed encephalitis in the shorter intervals after inoculation, although it is possible that exhaustive search might have shown some present.

Immunity.—Regularity of signs of illness produced by intracerebral inoculations of the Snyder Hill strain offered a possibility of providing a good test for immunity in recovered or vaccinated dogs. Accordingly, 20 dogs that had been given Snyder Hill virus either subcutaneously, intravenously, intranasally, intraperitoneally, or exposed by contact, were given the Snyder Hill virus intracerebrally after recovery. In addition, 5 dogs that had been given a vaccine strain of egg-propagated virus, obtained from Lederle Laboratories,* later were given Snyder Hill virus intracerebrally. Fifty-eight uninoculated dogs were included as controls.

None of the dogs after previous infection with either virulent virus or egg-propagated virus showed neurological manifestations or death when given the adapted Snyder Hill strain intracerebrally, whereas 57 of 58 previously uninoculated dogs did (table 3).

* Through the courtesy of Dr. Victor Cabasso.

Discussion

A strain of distemper virus designated Snyder Hill was obtained from a dog with distemper and has been transferred intracerebrally through 35 serial passages. As reported in 1951,[4] inoculated dogs in initial transfers developed either the catarrhal form of distemper only, or they developed epileptiform convulsions more than 22 days after inoculation. Beginning with the fifth passage, neurological manifestations have appeared with regularity 6 to 16 days after inoculation. In a recent paper, Cabasso[5] reported similar results with this strain. On histopathological examination of brains from dogs with epileptiform convulsions, perivascular cuffing, nonsuppurative meningitis, and neuronal degeneration characterized the lesion produced by inoculated virus.

Ability to transfer distemper virus by intracerebral inoculations was reported by Puntoni in 1923[6] and again in 1924.[7] In 1951, Bindrich[8] reported a strain of distemper virus transferable in series by suboccipital inoculation of spinal fluid that produced encephalitis. In 1951, Mansi[9] also reported on a strain of distemper virus that produced encephalitis. Furthermore, Mansi made careful comparison of his strain with distemper virus and could find no antigenic difference. Both in cross-immunity and in neutralization tests the Snyder Hill strain also proved to be antigenically similar to four other strains of distemper virus, including two that had been egg-propagated and another from a dog showing encephalitis and hardened footpads.

Intracerebral inoculations produced epileptiform convulsions in 57 of 58 dogs after the fourth transfer, while in initial transfers only some dogs were affected. It appeared that virus underwent some form of adaptation to the brain by intracerebral transfer. Inoculations of virus by other routes after nine or more intracerebral transfers, however, produced epileptiform convulsions in only 15 of 59 dogs. Failure

Fig. 3—Photomicrograph of cross section of dog cerebellum. Lower magnification of figure 1 showing vacuolation (arrows) of the cerebellum of a dog that developed nervous manifestations 22 days after intracerebral inoculation and was destroyed one day later. Hematoxylin and eosin. x 80.

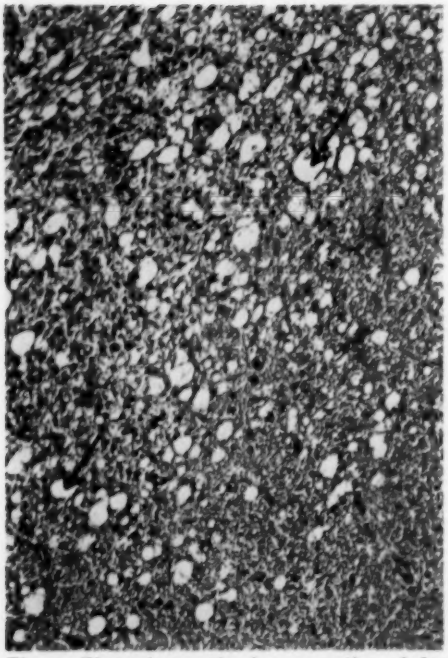

Fig. 4—Photomicrograph of cross section of dog cerebellum. Lower magnification of figure 2 showing marked lesions of demyelinating encephalitis (arrows) around the fourth ventricle of the brain of the dog that developed nervous manifestations 29 days after intracerebral inoculation and was destroyed two days later. Hematoxylin and eosin. x 160.

to achieve regularity in the production of encephalitis, by routes that would simulate natural means of transmission, may be caused by other factors as well as by the type of virus.

It is interesting that dogs recovered from the catarrhal form of distemper or given egg-adapted virus are fully immunized to intracerebral inoculations of the Snyder Hill strain. Since some dogs show almost inapparent illness following inoculation, intracerebral inoculation of the Snyder Hill strain provides a good method of testing immunity to distemper.

SUMMARY

An agent, designated as Snyder Hill, from a dog showing signs of generalized distemper infection only, has been transferred in series by intracerebral inoculation into other dogs. This agent was identified with distemper virus by cross-immunity and neutralization tests with established distemper strains. Thus far, 35 serial intracerebral transfers have been made. In initial passages, some inoculated dogs developed epileptiform convulsions after more than 22 days. Beginning with the fifth passage and in all subsequent transfers, neurological signs have appeared regularly six to 16 days after inoculation. Histological studies of affected dogs showed an encephalitis. Perivascular cuffing, nonsuppurative leptomeningitis with variable accumulations of mononuclear cells, and neuronal degeneration were the principal changes found in dogs that showed nervous signs after the shorter incubation periods. In two dogs that showed nervous manifestations after 22 and 29 days, there was also demyelination as evidenced by vacuoles and gitter cells in the white matter of the cerebellum and inclusion bodies in glial cells. After the fourth passage, all except 1 dog inoculated intracerebrally with adapted virus showed epileptiform convulsions or died without observed neurological signs. Only a fourth of the dogs that were inoculated by other routes showed these signs.

Apparently a form of distemper virus has been procured, and a method of inoculation found, that would regularly produce encephalitis in dogs. Dogs recovered from the generalized form of distemper induced by the Snyder Hill strain or by an attenuated egg strain were fully immune to intracerebral inoculations with the Snyder Hill strain.

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An Epizootic of Equine Infectious Anemia in Race Horses in Mexico

M. SCHERAGO, D.V.M.; GERARDO VARELA, M.D.; and ALINE S. HOFER, D.V.M.

Mexico, D. F.

AT THE invitation of the Hipodromo de las Americas in Mexico, the senior author arrived in Mexico on Aug. 3, 1954, to participate in an investigation of an epizootic among the horses at the track. The disease had apparently been prevalent at the track for over two years and had been diagnosed as babesiosis or anaplasmosis, or both.

HISTORY OF THE OUTBREAK

According to Dr. Alfonso Arzave, attending veterinarian, the first cases of the disease were observed in June, 1952, at two stables. Signs observed were fever, depression, anorexia, congestion, and slight jaundice and, occasionally, hemoglobinuria. The clinical and laboratory diagnosis was equine piroplasmosis. The animals were treated with tripaflavine, aralen and acaprin and they rapidly improved. However, after about two weeks, some of them suffered relapses and these were again given specific and supportive treatment in an attempt to speed recovery.

At the beginning of 1953 the outbreak seemed to be under control, but in September, 1953, new outbreaks appeared in animals quartered in other stables. These outbreaks seemed to coincide with the arrival of horses from a ranch where the animals had spent a rest period. On this ranch, 12 to 13 colts had reportedly fallen ill and died with similar symptoms.

Since then the disease has extended to many stables, and the above mentioned treatment, supplemented with arsenicals, bismuth salts, aureomycin,® chloromycetin,® sulfanilamide, enterovioforme, vitamins, and liver extracts, has failed to give satisfactory results. The animals apparently responded but suffered relapses at more or less regular intervals. The laboratory has reported the finding of *Babesia equi* and also "Anaplasma ?" in the blood smears obtained from the diseased horses.

The first horse presented for necropsy was Tea Pal. Findings were emaciation, slightly yellowish corneas and mucous membranes, subcutaneous gelatinous exudate, marked congestion and hemorrhage (petechiae to suffusions) of most of the abdominal and thoracic organs, and red infarctions in the ventricles. No parasites were found in smears from the blood and from the splenic pulp.

Early in 1954, another form of the disease began to appear. The main sign was a watery, nonfetid, persistent diarrhea which was resistant to all treatment. Other signs included anorexia, loss of weight, weakness, and depression. Usually there was no fever. The attacks of diarrhea alternated with periods when the feces were more or less normal but were covered with mucus. Some animals developed edema of the genitalia. The laboratory reported finding *Babesia equi* and "Anaplasma ?" in blood smears from these horses.

On June 30, 1954, 2 horses were given experimental inoculations: one, Time Gone, with blood obtained from Short Thorn, a horse with the fever-type of disease and a temperature of 106.3 F. on the day the blood was taken; the other, Dukery, with blood from H. Bone, a horse with the diarrhea-type of disease. Time Gone developed the fever-type of disease after seven days, while Dukery appeared to develop only a slight rise in temperature. Hence, on July 16, Dukery was inoculated with blood obtained from Time Gone.

There was no laboratory record on the presence or absence of parasites in Short

From the Department of Bacteriology, University of Kentucky, Lexington (Scherago), Instituto de Salubridad y Enfermedades Tropicales, Mexico, D. F. (Varela), Escuela Nacional de Medicina Veterinaria, Mexico, D. F. (Hofer).

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Thorn's blood prior to its use; however, blood smears obtained from Time Gone on July 13 and 14 showed the presence of *Babesia equi*. On July 26, Time Gone died and on necropsy the findings were similar to those in Tea Pal.

When Dukery failed to react to the injection of blood from H. Bone, and also from Time Gone, he was fed a suspension of feces from H. Bone. He continued to be refractory. On July 30, he was inoculated with blood obtained from Crisbone, a horse with the fever-type of disease and a laboratory report of "anaplasmoid" forms in his blood. On day nine, his temperature was 102.2 F., on day 13 105.8, then it gradually dropped to normal (100.2) by day 19. He had remissions from August 25 to September 10, and from September 25 to 30 when he died. At necropsy, the lesions were similar to those of Time Gone. No parasites were found in the blood.

On the basis of symptomatology there were two groups of horses, "F" those suffering primarily from fever and "D" those with marked watery, nonfetid diarrhea.

There apparently had been more than 70 horses with the fever-type, with at least 15 deaths, and 5 or more with the diarrhea-type, with no deaths, in a little more than two years. As our study began, there were some 20 horses with the fever-type and 5 with the diarrhea-type. *Babesia equi* or Anaplasma, or both, had been reported as responsible for both types of the disease.

Typical Case Histories

Since the case histories were similar for the horses in each of the two groups, we are presenting a typical one for each, case 1 (Blue Betty) an F horse that died and case 2 (Neddie's Pal) a D horse. Since no deaths occurred among the D horses, we are presenting a brief necropsy report on a D horse (case 3) that was destroyed.

Case 1.—Blue Betty (F type) was 3 years old; the date of onset is not known. Her temperature from July 1, 1954, until her death on August 14, fluctuated between 100.2 and 105.8 F. On August 6, she appeared dull, with head lowered. She refused grain but ate some alfalfa and drank more water than usual. Her feces and urine appeared normal but the amount of urine was increased. Urticarial spots, about 2 by 1 cm., were scattered over her sides and back, with edema of the ventral surface of the body, particularly toward the anterior portion and around the joints of all four legs. Her gait, particularly the hindlegs, was unsteady. The mucous membranes of the mouth and nose were slightly icteric, as were the conjunctivas. There were a few petechiae under the tongue and on the mucous membrane of the vagina. The temperature was 104.2 F., pulse 80, and respiration 20. No ticks were found on this animal or in her stall.

She received 26 injections of aralen between July 2 and August 10 and on August 5, 6, and 7 injections of aricil also. Blood smears taken daily from August 6 to 14 revealed no parasites. At necropsy, about two hours after death, the findings were not unlike those described for Tea Pal.

Pieces of various organs were frozen for future study. Inoculations from these organs (prior to freezing) into egg medium and nutrient broth and streaks on endo and SS agar plates yielded no significant microorganisms. The renal calyx material also proved negative.

Microscopic examination of the various organs revealed marked congestion and hemorrhage in most; cloudy swelling and necrosis of the hepatic cells; fatty degeneration and cloudy swelling of the renal tubules, many of which were necrotic, and infiltration of lymphocytes and plasma cells around the blood vessels and the glomeruli; and marked hemosiderosis in the spleen and liver.

Case 2.—Neddie's Pal (D type), a 4-year-old gelding, won a race Dec. 12, 1953. On January 4 or 5 he started having severe diarrhea, had a temperature of 100.4 F. and refused feed for a week. Since then he has eaten a little but has had alternating periods of diarrhea and of normal bowel movements, the latter once lasting two months. He lost approximately 100 kg. in weight. During the periods of diarrhea, there was edema of the hind legs below the stifle. At first, there was pronounced tenesmus with constant fluid evacuations. Even when the bowel movements were normal, there was tenesmus and some liquid at the end of the evacuation. The feces, when normally formed, were always

coated with a slight film of mucus but never any hemorrhage. During periods of intense diarrhea, his muzzle was cold, his mouth dry with petechiae in the mucous membrane and blisters in the gums and tongue. The urine was dark and was voided with tenesmus.

Of the various medications which this animal received, none proved efficacious.

At one time, the laboratory reported the isolation of "*Shigella paradysenterica* Flexner Vi Newcastle" from the feces; at another, the finding of *B. equi* in the blood smears and, on June 18 reported, "stained blood smears showed no protozoa."

On August 5, he appeared to be in fair condition except for the marked diarrhea. His appetite was good, there were no signs of colic, but there were frequent and copious evacuations of fluid and sometimes gaseous, but not fetid, feces. The walls of the stall were almost completely feces stained from the height of the horse to the floor. No intestinal parasites nor ticks were found.

While being observed and variously treated daily for six weeks, the feces were well formed but were coated with mucus. His maximum temperature was 102.2 F. and was high only during periods of diarrhea. On September 14, there was no change in the condition of the animal.

Case 3.—Dover Sky (D type), a 4-year-old gelding, had a persistent diarrhea since March, 1954, at which time the laboratory reported finding *B. equi* in his blood. Clinically, his case was much like that of Neddie's Pal, except that he became extremely emaciated. When killed, on September 8, the necropsy findings were extreme emaciation, pale mucous membranes, deep yellow subcutaneous tissues, icteric peritoneum, hemorrhagic lymph nodes, hemorrhage of the various organs, but not as marked as in F horses except in the mucous membrane of the small intestines where there were large areas of hemorrhage (as long as 15 cm.), and pink marrow in the long bones.

The histopathology revealed marked congestion and hemorrhage in most organs; marked fatty degeneration of the renal tubules; cloudy swelling and fatty degeneration of the hepatic cells; and marked hemosiderosis in the spleen and liver.

Laboratory Studies

Examination of Stained Blood Smears.—Since the laboratory reports prior to our study stated that blood smears from many of the horses revealed the presence of "*Babesia equi*" or "Anaplasma ?," or both, blood smears were obtained, some from the ear and some from inside the lower lip, from all horses that showed signs associated with F or D types. Included were all the survivors whose blood had been previously reported to contain Babesia or Anaplasma. The smears were stained with Giemsa's stain and examined. No structures resembling either *B. equi* or Anaplasma or any other parasites were found. In practically all smears Howell-Jolly bodies were occasionally found in the erythrocytes. Most of the smears showed anisocytosis, poikilocytosis, and polychromatophilia.

Daily temperatures and smears were taken from all the horses that had been examined previously and from all new cases. Any horse that showed a fever had his blood examined immediately, and then every six hours as long as the temperature remained elevated. Smears were taken each morning and evening until a relapse occurred. All were negative for parasites but, again, practically all smears from the sick horses showed occasional Howell-Jolly bodies, anisocytosis, poikilocytosis, and polychromatophilia.

Bacteriological Studies.—Blood cultures from both groups of horses, incubated both aerobically and anaerobically, yielded no significant results.

Fecal cultures yielded no pathogenic microorganisms. Smears, stained for acid-fast bacteria, were negative.

Examination for Fecal Microparasites.—Frequent fecal examinations showed no protozoa or other microparasites.

Tuberculin Test.—Intradermal tests, with Old Tuberculin, on the 5 D horses were negative.

Animal Inoculation.—Failure to find microparasites or other microorganisms that might account for the conditions and failure to find the species of ticks (*Dermacentor reticulatus* and *Rhipicephalus evertsi*) usually associated with the transmission of equine babesiosis, plus the absence of any record of a proved outbreak of equine anaplasmosis, particularly on this hemisphere, made the diagnosis of babesiosis or anaplasmosis untenable. The symptomatology, pathology, unsanitary environment, and a history of having used "the common syringe" for the injection of medication into several horses at the stables, indicated a diagnosis of equine infectious anemia. Nevertheless, the daily search for blood parasites continued and plans were made to carry out animal inoculations and other tests.

Differential Diagnosis by Animal Inoculation.—The differential diagnosis of equine infectious anemia is difficult. Since many consider the successful transmission of the disease to healthy horses[5] the only reliable method, we decided to

inoculate horses with blood from both the F and D horses. Of nonsolipeds, the pig is considered to be the most susceptible to the virus of equine infectious anemia. Hence, we decided to inoculate pigs. Since splenectomized donkeys have been reported to be especially susceptible to infection with *B.equi*, we planned to inject both splenectomized and nonsplenectomized donkeys. However, attempts to splenectomize donkeys proved unsuccessful so nonsplenectomized donkeys were used.

The animals used for inoculation were obtained from farms near Mexico City. Small pigs (8 lb.) were obtained since young pigs might be more susceptible than older ones. The horses were stabled at the School of Veterinary Medicine, about five miles from the Hipodromo de las Americas; the pigs and donkeys at the Instituto de Enfermedades Tropicales, about four blocks from the School of Veterinary Medicine.

For the animal inoculations two pools of blood were used, one from a group of 7 F horses and the other from a group of 5 D horses. Ten minutes prior to bleeding, each horse was given 2 ml. of a 1:1,000 dilution of adrenalin intravenously in an attempt to force the spleen to shed any microparasites that might be present there into the circulating blood.

Each pool of blood was divided into two portions. The plasma from one portion from each pool was filtered through a sterile Seitz filter. The filtered plasma from the F pool was divided into two portions. One portion was mixed with a filtrate obtained by triturating the frozen and thawed viscera from 1 F horse (Blue Betty) and filtering it through a Seitz filter. The materials injected into each animal and the reactions obtained are given (table 1).

Horse Inoculations.—Before the horses were inoculated and daily thereafter, blood smears were taken and examined for parasites, but none was found.

Horse M, which received 10 ml. of whole F blood, developed a temperature of 103.1 F. on postinoculation day 15, followed by 101.8, 102.2, 104.0, and 102.2 F. It was then normal for two days, then was 103.8 on day 22, then again normal for two days. This animal had relapses between days 26 and 31, days 44 and 45, days 60 and 63, and days 74 and 77 after inoculation. On postinoculation day 47, the erythrocyte count was 4,500,000 and the sedimentation rate was: 10 min.—139, 20 min.—154, and 24 hr.—165. By November 26 (day 85) the animal was prostrate so was killed. The necropsy findings were similar to those found in Ten Pal. The marrow of the long bones was dark yellow and gelatinous.

Microscopic examination revealed marked congestion and hemorrhage in most organs; marked cloudy swelling and fatty degeneration in the kidneys and liver; and marked hemosiderosis in the spleen.

Horse G, which received 10 ml. of whole D blood, developed a temperature of 101.5 F. on postinoculation day 15, then 103.8, 106.2, 102.2, and 101.3, returning to normal for five days with relapses between days 25 and 26, days 29 and 33, days 46 and 49, days 59 and 60, and days 78 and 88. By postinoculation day 85, the animal had developed an unsteady gait and two days later developed diarrhea. On day 88 the erythrocyte count was 3,770,000 and the sedimentation rate was: 20 min.—124, 60 min.—142, and 24 hr.—148. On November 30 (day 89), the animal was moribund, so it was killed. The necropsy findings

TABLE 1—Animal Inoculations

Identification	Material injected (Sept. 2, 1954)	Reaction
HORSES		
M	10 ml. whole F blood	Fever in 14 days, moribund in 86 days
G	10 ml. whole D blood	Fever in 18 days, moribund in 90 days
C	10 ml. mixture of equal parts of filtered F plasma, filtered D plasma and filtered BB triturated tissue	Fever in 10 days, died in 59 days
DONKEYS		
SN	5 ml. whole F blood	None
164	5 ml. whole D blood	None
153	5 ml. equal parts filtered F plasma and filtered BB tissue	None
156	5 ml. filtered D plasma	None
PIGS		
139	5 ml. whole F blood	Purpuric spots on elbows and paralyzed in hindlegs by day 4; died on day 12
145	5 ml. whole D blood	Purpuric spots on chin, abdomen, elbows, etc., and wobbly, swollen hindlegs by day 4*
140	5 ml. filtered F plasma	Purpuric spots on left leg by day 4*
148	5 ml. filtered D plasma	Purpuric spots on right foreleg and wobbly hindlegs by day 4*
TR	5 ml. filtered BB tissue	Purpuric spots in inguinal region and perineum by day 4*
PN	5 ml. pooled whole blood from two normal horses injected 7 days later than the other pigs. (Sept. 9, 1954)	Outlived its mates; no record available of time of death

*All pigs were reported (by G.V.) to have died in from 14 to 16 days; F—fever horses; D—diarrhea horses; BB—tissue filtrate from Blue Betty was used.

were similar to those found in horse M, except that the bone marrow was pink.

Horse C, a colt, received 10 ml. of a mixture of equal parts of filtered F plasma, filtered D plasma and filtered triturated tissue from Blue Betty (1 of the F horses that died) on Sept. 2, 1954. Its temperature on the day of inoculation was 101.7 F. The next two days it was 102.6, on postinoculation days 3 to 7 it was 102.2 or less but on days 8 to 11 it was 103.1 to 103.3. It dropped to 102.2 on day 12, rose the next day to 104.2, remaining elevated for seven days (105.4, 105.8, 105.4, 104.7, 104.4, 102.4, and 103.6). This animal had relapses on days 28 to 31 and on days 46 to 52. On October 18 (day 46) the erythrocyte count was 5,040,000 and the sedimentation rate was: 10 min.—120, 20 min.—138, and 24 hr.—157. The colt died on day 52. The necropsy findings were similar to those of horse G.

Donkey Inoculations.—The 4 donkeys inoculated (table 1) all appeared to be refractory.

Pig Inoculations.—Five pigs were inoculated (table 1) to determine whether the equine disease was transmissible to swine. A sixth pig, used as a control, was injected one week later with a pool of blood from 2 horses that had received no injections of any kind. Blood smears obtained from them at the time the blood samples were obtained showed no parasites.

In from four to five days after injection all the inoculated pigs developed purpuric spots on the skin (fig. 1) of the abdomen, legs, the perineal region or the inguinal region. These spots gradually faded and after a week were hardly noticeable. Three of the pigs, 139 (F blood), 145 (D blood), and 148 (filtered F plasma), also developed wobbly swollen hindlegs by postinoculation day 5 and the legs of pig 139 were completely paralyzed by day 6. The conjunctivas of all the pigs except TR (tissue filtrate from Blue Betty) and the control were pale by day 8. By day 13 the control pig still showed no lesions and appeared normal. Pig 139 died on day 12 and at necropsy showed only markedly congested abdominal lymph nodes. Histological sections of these glands, stained with hematoxylin and eosin, showed the same thing. The larynx, trachea, kidneys, and urinary bladder appeared normal. No petechiae were found.

The other 4 inoculated pigs were reported (G.V.) to have died by postinjection day 16 and to have showed emaciation and petechiae. The control pig outlived its mates, but no record is available of when it died.

Tissues from the pigs, which had been preserved by freezing, were thawed, triturated with physiological salt solution, and filtered through a Seitz filter. Five-milliliter amounts of the filtrate were injected subcutaneously into a second series of young pigs. All the pigs developed weakness, especially of the hindlegs, and diarrhea and died 14 days after they were injected. "The lesions were

TABLE 2—Results of Complement-Fixation Test

Date	Horse (No.)	Horse (Name)	Reaction*	Comments
12/18/54	1	Number 1	3+	.....
12/18/54	2	Number 2	1+	.....
12/18/54	3	Number 3	3+	.....
12/18/54	4	Number 4	4+	.....
12/21/54	5	Crisbone	2+	Onset July 29, 4 attacks; no relapse since Sept. 11; does not race well; exhausted after half the course.
12/21/54	6	Awrility	2+	Won two races recently.
12/21/54	7	Short Thorn	4+	Still periodic fever attacks; third in a race recently.
12/21/54	8	No Name	4+	Never known to have been sick.
12/28/54	9	Bandera Negra	2+	Last relapse October, 1954.
12/28/54	10	Alianza	3+	Had several attacks; died a few hours after blood was taken.
12/28/54	11	Camionero	1+	Had several acute attacks; died Jan. 22, 1955.
12/28/54	12	Polly D.	1+	Became sick on Dec. 11, 2 acute attacks; died Jan. 17, 1955.
1/10/55	13	Tormentosa	1+	Died shortly after sample was taken.
1/10/55	14	Soot and Sand	2+	Last attack October, 1954.
1/10/55	15	Polly D.	1+	See number 12.
1/10/55	16	Radamés	4+	Still periodic fever attacks.
1/10/55	17	Alacrán	4+	Just recovered from fever attack when blood was taken.
1/10/55	18	Pony	3+	Never known to have been sick.
1/14/55	19	Gran Danés	1+	Last attack months ago; won race recently.
1/14/55	20	Short Thorn	4+	See number 7.
1/14/55	21	Dona Trampa	4+	No relapse recently.
1/14/55	22	Aparecido	4+	Just recovered from a fever attack when blood was taken.
1/14/55	23	Maldito	3+	Last attack Oct.-Nov., 1954.
1/14/55	24	Polly D.	1+	See numbers 12 and 15.

* 1 + very slight or no inhibition of hemolysis = negative.
2 + slight inhibition of hemolysis = doubtful.
3 + partial inhibition of hemolysis = positive.
4 + complete inhibition of hemolysis = positive.

not as typical as those which we had seen in the first series." (G.V.)

The tissues from the second series of pigs were preserved by freezing at low temperatures for three days, were then removed and treated as those from the first series. The filtrate was injected into a third series of 3 young pigs which had received, prior to the injection of the filtrate, 5 ml. of anti-hog cholera serum, obtained from the U. S. Department of Agriculture. None of these pigs suffered any ill effects. Unfortunately, no nonimmunized control pig was injected with the filtrate; nor were any of the 3 pigs challenged, in addition, with blood from the sick horses. We hope to have an opportunity to repeat this experiment with appropriate controls.

Serological Studies.—Following the inoculation experiments, it was decided to try the complement-fixation test for equine infectious anemia recently described by Altara, Serra, and Guarini.[1] These authors found that the serums of horses with the subacute or chronic form of equine infectious anemia and those from horses that are carriers of the virus give positive reactions, while the serums from animals with the acute form may give negative reactions. According to Prof. Serra,[5] during the febrile attack the reactions may also be negative, but when the fever subsides the serum again becomes positive.

Antigen and a known positive and a known negative serum were obtained from Prof. Serra. Five series of tests (Dec. 18, 21, and 28, and Jan. 10 and 14) were done on serums from 21 horses (table 2). Serums from 12 horses (57%) gave complete (4+) or nearly complete (3+) inhibition of hemolysis, which we considered a positive reaction; 4 (19%) gave a partial inhibition (2+), which we considered a doubtful reaction, and 5 (24%) gave little or no inhibition (1+), which we considered a negative reaction. From 1 horse, Short Thorn, samples were obtained on Dec. 21, 1954, and on Jan. 14, 1955, both samples giving a 4+ reaction. From another horse, Polly D, samples were obtained on Dec. 28, 1954, Jan. 10 and 24, 1955; all gave a 1+ reaction.

Duplicate samples of serums from 6 horses (7, 12, 14, 17, 18, and 19) were sent to Dr. Serra to be tested in his laboratory. Three of the serums (7, 17, and 18) were positive, and two (12 and 19) were negative at both laboratories while one (14) was found to be doubtful by us and negative by Dr. Serra.

DISCUSSION

What impressed us most in the history of the outbreak was the diagnosis of anaplasmosis, since this condition had never been authentically reported in horses. Balfour[2] reported finding anaplasmosis in donkeys in the Sudan, and Brion[3] described an outbreak in horses in the Haute-Savoie region in France. Both based their diagnosis on finding, in the blood of the horses, structures which they thought resembled Anaplasma. Brion differentiated these structures from Jolly bodies by reproducing the disease in an experimental horse with erythrocytes obtained from a diseased horse. His claim would have been more convincing had he injected a control horse with filtered plasma, since the symptoms he described could well have been those of equine infectious anemia. He presented no photomicrographs of the structures which he considered to be Anaplasma. Brion also quotes Yakimoff and Koselkine[10] as having observed in Russian Turkistan "anaplasmosis in two horses, in one associated with *Nuttalia equi* and in the other alone." Brion comments that it is "not certain that these authors, who described neither the disease nor the parasite, have not seen the anaplasmoid forms of *Nuttalia equi*, studied by Carpano and described earlier by Balfour." We felt, therefore, that if the diagnosis of anaplasmosis in the race horses at the Hipodromo de las Americas could be substantiated, we would have evidence of a new equine disease, at least on this hemisphere. Also, in view of the report of the Russian workers finding Anaplasma in association with *Nuttalia equi*, the laboratory reports of the presence of both *B. equi* and Anaplasma in the blood smears from some of the horses at the track were of special interest.

Although the signs and pathology of the F horses were not incompatible with a diagnosis of babesiosis in horses or anaplasmosis resembling the disease in cattle, our failure to find parasites in any of the blood smears from the sick animals made such a diagnosis untenable.

When we examined slides in which the presence of Anaplasma had been reported, the suspected structures were in some cases Howell-Jolly bodies and in others, artefacts. We were not afforded an opportunity to examine any of the slides on which *B. equi* had been reported. However, blood smears from 1 horse did show the cloverleaf structure of *B. equi*. This was the experimental horse that had received an injection of blood from one of the F horses, but there was no laboratory report on the presence or absence of *B. equi* in the donor's blood. We can not know, therefore, whether the injected horse was infected from the inoculation or was a car-

rier of the parasites. At no time were we able to find any parasites in the donor's blood. Curiously, a second horse injected with blood from the same donor nine days after the latter first developed fever showed no parasites although blood smears were examined daily until he died.

The case of the D horses was even more baffling. Their symptoms resembled neither equine babesiosis nor anaplasmosis in cattle yet both *B. equi* and Anaplasma had also been reported in their blood. We failed to find these parasites. The question, then, was whether there were two types of diseases or two kinds of manifestations of one disease.

Bacteriological studies of the feces and blood from both groups of horses revealed no pathogenic microorganisms, nor were pathogenic protozoa detected in the feces. Tuberculin tests on 5 of the D horses were also negative.

The history, signs, pathology, and negative laboratory findings for babesiosis and anaplasmosis warranted a tentative diagnosis of equine infectious anemia. This diagnosis was confirmed when we transmitted the disease to horses with blood from F horses and D horses and also with filtered plasma from both.

Whether the D horses were also victims of the virus of infectious anemia may be open to some question. The experimental horse which was injected with blood from this group of horses developed symptoms and pathological changes that were characteristic of those of the F group. Yet it did finally develop diarrhea two days before it became moribund. It is possible that the diarrheal condition of the D group was due to some other cause and that these animals were, in addition, merely carriers of the virus of infectious anemia, which accounted for the development of the F type of disease in the experimental horse that received their blood. On the other hand, Dreguss and Lombard [6] quote Carré and Vallée [5] as having reported diarrhea as one of the symptoms of equine infectious anemia, as have Stein [9] and Hutyra, Marek, and Manninger.[7]

Our attempt to transmit the disease to normal donkeys was unsuccessful.

Whether we succeeded in infecting pigs with the blood from the diseased horses may be open to question. All 5 of the inoculated pigs developed purpuric spots within three to four days after they were injected and 3 of them developed wobbly hindlegs by the fourth day, while the uninoculated control pig remained free of these symptoms for at least 14 days after its mates had been injected and at least ten days after they had begun to develop symptoms. The second series of pigs which were inoculated with filtered triturated tissue from the first series developed symptoms of weakness, especially of the hindlegs, and diarrhea and died within 14 days after injection. When the filtered triturated tissue from the second series was inoculated into a third series of young pigs previously immunized with anti-hog cholera serum, none of the latter was affected. Unfortunately, these pigs were not challenged, in addition, with blood from the diseased horses. This raises the quesion as to whether the first series of pigs reacted to the virus of equine infectious anemia and then contracted hog cholera or whether the pigs were already infected with the hog cholera virus when they were inoculated with material from the diseased

Fig. 1—Purpuric spots on a pig injected with filtered plasma.

horses and, hence, the reactions which we observed were due to hog cholera virus and not to the virus of equine infectious anemia. It is important to emphasize, however, that these pigs were observed for seven days before they were injectd with the blood from the diseased horses and that during this period there were no signs of hog cholera, also, that the control pig remained free of symptoms for at least ten days after they appeared in its inoculated mates although it was kept together with them in the same quarters.

If the virus of equine infectious anemia was responsible for the signs and the death of the first series of pigs, failure of the hog cholera-immunized pigs to become infected with material from the second series of pigs might have been due to the inability of the virus of equine infectious anemia to survive passage through a foreign host (pig). At any rate, the results of the primary inoculations would appear sufficiently significant to warrant repeating the experiment by injecting young hog cholera-immunized pigs or suckling pigs from a hog cholera-immune sow with blood from horses with equine infectious anemia. This we plan to do.

Our experience with the complement-fixation test of Altara, Serra, and Guarini[1] is too limited to enable us to assess its dependability for the diagnosis of equine infectious anemia.

SUMMARY AND CONCLUSIONS

An outbreak of a disease that had been prevalent in the race horses at Hipodromo de las Americas in Mexico for over two years and that had been diagnosed as babesiosis or anaplasmosis, or both, was studied. Two types of conditions were found: one characterized by intermittent periods of fever (F); the other by marked, watery, nonfetid diarrhea (D).

Microscopic examination of hundreds of Giemsa-stained blood smear preparations from all the diseased horses of both types failed to reveal any parasites. Gastrointestinal parasites were not found. Blood cultures and fecal cultures yielded no significant results. Tuberculin tests (intradermal) on 5 horses with diarrhea were negative.

The absence of microparasites or other microorganisms and ticks (*Dermacentor reticulatus* and *Rhipicephalus evertsi*) made the diagnosis of babesiosis and anaplasmosis untenable. The symptomatology, pathology, the unsanitary environment, and the use of "the common syringe" all pointed to equine infectious anemia as an alternative diagnosis. This diagnosis was confirmed by the transmission of the disease to horses with blood from F horses and D horses, and with a mixture of Seitz-filtered plasma from both and filtered triturated tissue from an F horse.

Donkeys injected with whole blood and with filtered plasma from both groups of horses were unaffected.

Young pigs that were similarly injected developed purpuric spots and swollen, wobbly, paralyzed hindlegs in from four to six days after injection. A control pig had not developed these signs 14 days after its mates had been injected and a week after it had received pooled blood from 2 normal horses. All the inoculated pigs died by the sixteenth day following inoculation of the experimental pigs. The significance of the reaction in these pigs and others subsequently injected is discussed.

The complement-fixation test of Altara, Serra, and Guarini gave positive reactions with 57 per cent of the serums tested, doubtful with 19 per cent, and negative with 24 per cent.

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Nematocidal Screening Tests of Organic Phosphorus, Nitrofuran, Cadmium, and other Compounds Against Horse Strongyle Larvae *in Vitro*

NORMAN D. LEVINE, Ph.D.; VIRGINIA IVENS, B.S.;
MARLIN D. KLECKNER, B.S.; JEAN K. SONDER, M.S.

Urbana, Illinois

FOR SEVERAL years, a screening program for potential nematocides has been carried out at the University of Illinois in which various compounds have been tested for activity against horse strongyle larvae and eggs in feces. The present paper reports the results obtained with eight organic phosphorus compounds, nine cadmium compounds, five nitrofurans, 13 heterocyclic compounds, 22 other cyclic compounds, three aliphatic compounds, and one antibiotic.

The technique used has been described by Levine.[1,2] Briefly, the compound to be tested was premixed with talcum powder to give it body and then mixed with fresh horse feces containing strongyle eggs. The mixture was placed in a wide-mouthed glass jar, and the jar was placed in a dark cupboard at room temperature for a week. At the end of this time, the feces were examined for larvae, using the Baermann technique. If no larvae could be found, the compound was considered active. Untreated controls were also set up and examined. Each compound was set up in concentrations of 0.01 M, 0.005 M, 0.0025 M, 0.001 M, and in lower concentrations if indicated by the results with the higher ones.

Well over 95 per cent of the eggs and larvae in the feces were those of small strongyles; the remainder were those of Strongylus spp. No significant difference was noted between the results with large and small strongyles.

RESULTS

The compounds listed (table 1) killed or prevented the development of all larvae in feces in the concentrations indicated unless otherwise stated. For a few compounds, two different active concentrations are given; this is done only when the results of repeat tests differed. One reason for the observed variation may have been incomplete mixing of the compound with the feces, which permitted larvae to hatch without exposure to the compound.

The lowest active concentrations are given both in moles and as percentages. The former permits equimolecular comparisons to be made between related compounds, while the latter indicates the relative weights of compound and feces.

DISCUSSION

To the organic phosphorus compounds listed herein should be added tetraisopropylpyrophosphate, tetrabutylpyrophosphate, and diethyl phosphoric acid, which Levine[3] found inactive against horse strongyle larvae at a concentration of 0.01 M (0.346 %, 0.402 %, and 0.154 %, respectively), triphenylmethylphosphonium iodide, which Levine[2] found active at 0.0005 M (0.025 %), and tetraethylpyrophosphate, which Levine[11] found active at a concentration of 1.0 per cent but not at 0.1 per cent. In contrast with all the compounds tested earlier except the phosphonium iodide, the present series exhibited marked activity. Although most of them were thiophosphate esters, replacement of an oxygen by sulfur is not necessary for high activity nor does it always produce such activity. The most active compound in the whole group was the phosphonate, Bayer L 13/59, which does not contain sulfur. Furthermore, the only difference between the two phosphorothioates, Dow ET-14, which was inactive at a concentration of 0.105 M, and Dow ET-15, which was active at 0.00078 to 0.005 M, was the replacement of a methoxy group attached to the phosphorus atom in the former by an amino group in the latter.

It is of great interest that McGregor, Radeleff, and Bushland[4] found that when fed to cattle infested with Hypoderma, diazinon and Bayer L 13/59 gave excellent kill of the grubs present along the back, while chlorthion killed approxi-

From the Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine, and Agricultural Experiment Station, University of Illinois, Urbana (Levine and Ivens), and Animal Disease and Parasite Research Branch, Agricultural Research Service, U. S. Department of Agriculture (Kleckner and Sonder).

TABLE 1—Compounds that Killed or Prevented Development of all Horse Strongyle Larvae in Vitro in the Concentrations Indicated

Compound	Lowest active concentration (Molar)	Lowest active concentration (%)
ORGANIC PHOSPHORUS COMPOUNDS		
O,O-dimethylhydroxy-2,2,2-trichlorophosphonate (dipterex; Bayer L 13/59)[1]	0.0001	0.004
	0.00025	0.007
S-(1,2-dicarbethoxyethyl) O,O-dimethyl dithiophosphate (malathion)[2]	0.005	0.02
O-(3-chloro-4-nitrophenyl) O,O-dimethyl thiophosphate (chlorthion) (90 %)[2]	0.0019	0.06
3-chloro-4-methylumbelliferone O,O-diethylthiophosphate (Bayer 21/199)	0.0074	0.27
(quite finely ground)[2]	0.0025	0.08
O,O-diethyl-O-[2-isopropyl-4-methylpyrimidyl-(6)] thiophosphate (diazinon)[2]	0.105 inactive	2.5 inactive
O,O-dimethyl 2,4,5-trichlorophenyl phosphorothioate (Dow ET-14)[4]	0.00078	0.03
O-methyl-O-2,4,5-trichlorophenyl phosphoramidothioate (Dow ET-15)[4]	0.005	0.16
	0.008	0.31
triphenyl isopropyl phosphonium bromide[5]		
CADMIUM COMPOUNDS		
cadmium oxide	0.0025	0.032
	(0.001 partial)	(0.013 partial)
cadmium carbonate	0.01 inactive	0.172 inactive
cadmium sulfide	0.01 inactive	0.145 inactive
cadmium bromide	0.001	0.034
cadmium chloride	0.0025	0.046
cadmium sulfate	0.01	0.208
cadmium acetate	0.001	0.027
cadmium nitrate	0.001	0.031
	(0.0005 partial)	(0.015 partial)
cadmium anthranilate[6]	0.01	0.39
NITROFURANS		
N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone (furazolidone)[7]	0.025	0.57
	(0.0025 partial)	(0.057 partial)
N-(5-nitro-2-furfurylidene)-1-aminohydantoin[8]	0.01	0.226
5-nitro-2-furaldehyde pyridinium acethydrazone chloride[3]	0.01 inactive	0.295 inactive
5-nitro-2-furaldehyde-4-(3-diethylaminopropyl) semicarbazone hydrochloride[5]	0.01 inactive	0.348 inactive
5-nitro-2-furaldehyde semioxamazone[5]	0.01 inactive	0.226 inactive
OTHER HETEROCYCLIC COMPOUNDS		
NITROGEN IN RING		
N-bromosuccinimide[6]	0.01 inactive	0.178 inactive
2-chloro-4,6-dimethylnicotinonitrile[6]	0.01	0.167
	0.05	0.084
8-hydroxyl-1-methylquinolinium methylsulfate[6]	0.01 inactive	0.271 inactive
N-trichloromethylthio-Δ^4-tetrahydrophthalimide[6]	0.01 inactive	0.301 inactive
3,6-*bis*-(dimethylamino)-10-laurylacridinium bromide[6]	0.01 inactive	0.515 inactive
piperazine	0.01 inactive	0.861 inactive
2-(*p*-anisidino)-4-chloro-6-(β-diethylamino-ethylamino)-1,3,5-triazine[6]	0.01 inactive	0.351 inactive
OXYGEN IN RING		
2-nitro-1-furyl ethylene (Ortho HL 736)[7]	0.005	0.07
	(0.0025 partial)	(0.04 partial)
2,6-dimethylpyran-4-oxo-dimethylformamide (Geigy 23091)[2]	0.005	0.10
SULFUR IN RING		
2,5-dicarboxy-3,4-diphenylthiophene[8]	0.01 inactive	0.32 inactive
MIXED RINGS		
2-chloro-6-nitrobenzothiazole[6]	0.005	0.108
	(0.01 partial)	(0.215 partial)
tetraiodo-o-sulfobenzoic anhydride[6]	0.01 inactive	0.688 inactive
OTHER CYCLIC COMPOUNDS		
1,1,1-trichloro-2-hydroxy-2-phenylethane[6]	0.01	0.226
	(0.005 partial)	(0.113 partial)
α-chloro-3,4-dihydroxyacetophenone[6]	0.01	0.187
1-(*o*-chlorophenyl)-2-nitropropene[6]	0.005	0.099
	0.01	0.198
	(0.005 partial)	(0.099 partial)
p-hydroxybenzaldehyde thiosemicarbazone[6]	0.01 inactive	0.195 inactive
1,4-*bis*-[*p*-(2-octyl)benzyldimethylammonium]-2-butyne dichloride[6]	0.01 inactive	0.618 inactive
p-nitrobenzene sulfonyl chloride[5]	0.01 inactive	0.22 inactive
ethylbenzene sulfonate[5]	0.01	0.19
benzene sulfon-*m*-anisidide[5]	0.01 inactive	0.26 inactive
2,5-dibromobenzene sulfonyl chloride[5]	0.01 inactive	0.34 inactive
sodium prehnitene sulfonate[5]	0.01 inactive	0.24 inactive
barium 3-nitro-4-hydroxybenzene sulfonate[5]	0.01 inactive	0.57 inactive
benzyl sulfon-*p*-toluide[5]	0.01 inactive	0.26 inactive
benzyl sulfonanilide[5]	0.01 inactive	0.22 inactive
benzyl sulfonethylanilide[5]	0.01 inactive	0.28 inactive
bis-(5-chloro-2-hydroxyphenyl)-methane (diphenthane-70)[9]	0.01 inactive	0.269 inactive
1,1-dichloro-2,2-*bis*-(*p*-nitrophenyl) ethylene[5]	0.01 inactive	0.339 inactive
N-(3,4-dichlorobenzyl)-*p*-chloroaniline[6]	0.01 inactive	0.287 inactive
3,5-dichloro-2-hydroxyphenyl sulfide[9]	0.01 inactive	0.356 inactive
diphenyl sulfoxide[8]	0.01 inactive	0.20 inactive
5-[bis-(3,4-dichlorobenzyl)amino]-isophthalic acid[6]	0.01 inactive	0.499 inactive
benzidene monosulfonic acid[5]	0.01 inactive	0.27 inactive
1-aminonaphthalene-3,8-disulfonate, monosodium salt[5]	0.008 inactive	0.25 inactive
ALIPHATIC COMPOUNDS		
potassium hydrogen amino methane disulfonate[8]	0.01 inactive	0.27 inactive
1,2-*bis*-(β-chloroethylsulfonyl) ethane[5]	0.01 inactive	0.28 inactive
diiodomethane disulfonate, dipotassium salt[5]	0.0025	0.13
ANTIBIOTIC		
filipin[8]		0.4 inactive

[1] Supplied by Chemagro Corp., New York, N. Y., through the courtesy of Dr. Rosemarie von Rümker; [2] Supplied by Dr. Willis N. Bruce, Illinois Natural History Survey, Urbana; [3] Geigy Agricultural Chemicals, New York, N. Y., Dr. C. C. Alexander; [4] Dow Chemical Co., Midland, Mich., Dr. J. E. Johnson; [5] Dr. Hess & Clark Co., Ashland, Ohio, Dr. Paul D. Harwood; [6] Sharp & Dohme, West Point, Pa., Dr. S. F. Scheidy; [7] California Spray-Chemical Corp., Richmond, Calif., Dr. Edward G. Batte; [8] Dr. C. S. Marvel, Dept. of Chemistry, Univ. of Illinois, Urbana; [9] Dr. David Gottlieb, Dept. of Horticulture, Univ. of Illinois, Urbana.

mately two thirds of them, and Bayer 21/199 was completely ineffective.

The first organic phosphorus insecticides to be developed, such as tetraethylpyrophosphate and parathion were much too toxic for warm-blooded animals to be considered for internal administration as anthelmintics. The lethal dose $(l.d.)_{50}$ for rats of tetraethylpyrophosphate is 0.85 mg. per kilogram of body weight,[5] and that of parathion is 4.0 mg. per kilogram of body weight.[6] More recently, compounds of relatively low toxicity have been developed. DuBois and Cotter[7] reported that the acute intraperitoneal $l.d._{50}$ of Bayer L 13/59 for rats, guinea pigs, and mice are 225, 300, and 500 mg. per kilogram of body weight, respectively, and that the compound is about half as toxic when given orally. According to the manufacturer, the acute oral $l.d._{50}$ of diazinon for rats is 220 to 750 mg. per kilogram. McGregor, Radeleff, and Bushland[4] fed 100, 50, 25, and 25 mg. per kilogram, respectively, of Bayer L 13/59, chlorthion, Bayer 21/199, and diazinon to cattle without apparent injury. In view of this relatively low toxicity for mammals and high toxicity for horse strongyle larvae *in vitro*, it is felt that further investigation of this group of compounds should disclose several practical and effective anthelmintics.

To these cadmium compounds should be added cadmium iodide, which Lævine and Ivens[8] found to be active against horse strongyle larvae in feces at a concentration of 0.00025 M (0.010 %). An abstract of this section of the present paper has been published.[10] These cadmium compounds were investigated because cadmium oxide had been introduced as an anthelmintic against *Ascaris lumbricoides* in swine.[9] It was found that while there is some correlation between solubility in water and anthelmintic activity against horse strongyle larvae, the correlation is not invariable. Cadmium carbonate and cadmium sulfide, which are insoluble or nearly so in water, were inactive at 0.01 M, the highest concentration tested; cadmium bromide, chloride, sulfate, acetate, iodide, and nitrate, all of which are very soluble in water, were active at concentrations ranging from 0.001 to 0.01 M. On the other hand, cadmium oxide which is insoluble, was active at 0.0025 M.

To the five nitrofurans listed herein should be added nitrofurazone (5-nitro-2-furaldehyde semicarbazone), which Lævine[3] found inactive at 0.01 M (0.198 %), the highest concentration tested. A large number of nitrofurans have been synthesized; several of them have marked activity against bacteria, and nitrofurazone is an effective coccidiostat for *Eimeria tenella* infections in chickens. It is felt that further study of this group might reveal some nitrofurans with greater anthelmintic activity than those so far tested.

Although their activity was not of a high order, 1,1,1-trichloro-2-hydroxy-2-phenylethane, α-chloro-3,4-dihydroxyacetophenone and 1-(*o*-chlorophenyl)-2-nitropropene deserve special mention. The first two are chlorinated phenylethane derivatives, while the third is a chlorinated phenyl propene derivative. A study of a larger series of related compounds might be rewarding. Such factors as position of the chlorine atom or atoms, length and type of the alkyl sidechain, and presence and position of other substituents might be considered in such a study.

Summary

In a study of the effect of 61 compounds on horse strongyle larvæ in feces, it was found that seven of eight organic phosphorus compounds, seven of nine cadmium compounds, two of five nitrofurans, four of 13 heterocyclic compounds, four of 22 other cyclic compounds, and one of three aliphatic compounds killed or prevented the development of the larvae at a concentration of 0.01 M or less. The antibiotic, filipin, was inactive at a concentration of 0.4 per cent. Compounds which were active at 0.001 M or lower were O,O-dimethylhydroxy-2,2,2-trichlorophosphonate (Bayer L 13/59), 0.0001 to 0.00025 M; O-methyl-O-2,4,5-trichlorophenyl phosphoramidothioate (Dow ET-15), 0.005 to 0.00078 M; cadmium bromide, 0.001 M; cadmium acetate, 0.001 M; and cadmium nitrate, 0.001 M.

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Radiographic and Histopathological Findings in the Bones of Swine Deficient in Manganese

G. M. NEHER, Ph.D.; L. P. DOYLE, D.V.M., Ph.D.; D. M. THRASHER, M.S.; M. P. PLUMLEE, Ph.D.

Lafayette, Indiana

IN GROWING swine a number of bony dystrophies, lamenesses, and spontaneous fractures occur which do not appear to be related to erysipelas, arthritis, or any other infectious disease. Indeed, the etiology of these conditions may seem very obscure since many of the droves of swine in which they occur are being fed what are presumed to be the best possible rations. It has been recognized for some time that pigs being raised on a ration composed chiefly of skimmed or whole milk, although they grow at a very favorable rate, are subject to a high incidence of spontaneous fractures. It is felt that a deficiency of some of the so-called trace minerals may be partially responsible for some hitherto unexplained skeletal dystrophies.

Manganese deficiency has been studied quite extensively in poultry and it, as well as choline deficiency, has been clearly incriminated as the cause of perosis or "slipped tendon" disease.[1] These authors have demonstrated that manganese deficiency caused a retardation or suppression of endochondral growth at epiphyseal cartilages and they believe that this may account for the characteristic deformities of the hock joints of birds with the disease. Bony dystrophies have resulted from manganese deficiency in rabbits,[2] rats,[3] and mice.[4]

The effect of manganese deficiency on impaired skeletal growth in swine was first reported by Miller *et al.*[5] in 1940. Thirty of 60 pigs raised on a diet deficient in manganese from weaning time (8 weeks) became lame when they weighed about 150 lb. The condition was characterized by pain, enlargement of the hocks, crooked legs, and enlargement of the distal ends of the radius and ulna. The mineral content of these bones, however, was within the range for normal swine. The condition failed to appear when the ration was supplemented with manganese sulphate in amounts which would bring the level of manganese to 50 to 60 parts per million (p.p.m.). The results of Keith *et al.*[6] confirmed these observations and also showed that the addition of vitamin D to the manganese-deficient ration did not prevent or relieve the condition.

In 1944 Johnson[7] reported favorable growth of pigs being fed rations which contained between 7 and 10 p.p.m. and he postulated that manganese deficiency would be unlikely to occur under natural conditions. Grummer *et al.*[8] in 1950 reported that the critical level of manganese for growing pigs seemed to be between 12 and 20 p.p.m., and a ration containing 500 p.p.m. caused a marked retardation in growth. Whereas the manganese requirements for swine are not clearly defined, the National Research Council's Committee on Swine Nutrition[9] recommends 20 to 40 p.p.m.

The purpose of this investigation was to make radiographic and histological examinations of the bones of the legs of swine which were maintained on manganese-deficient diets in an effort to reveal the cause of lameness, distortion of the legs, and retardation of growth. Comparative studies were made on litter mate pigs which were provided a ration adequate for manganese.

MATERIALS AND METHODS

Nineteen female pigs 3 weeks of age and weighing an average of 9.5 lb. were divided into two groups and housed indoors in pens with concrete floors. The first group (9 pigs) was fed a purified ration containing 0.5 p.p.m. of manganese; the second group (10 pigs) received the same ration but it was supplemented with 40 p.p.m. of manganese. The initial ration contained 24.5 per cent of protein and consisted of dried skim milk as a protein source; corn sugar; fiber (cellufluor, Chicago Dietetic Co.); corn oil; 0.5 per cent dried liver powder; adequate vitamins and purified minerals. During the growth period between 30 and 135 lb., the percentage of protein was lowered to 18 after which time it was lowered to 14 per cent. After the gilts weighed 170 lb., the ration was altered to provide 12 per cent of protein. Each feed mix was analyzed for manganese according to the A.O.A.C. procedure and the ration was adjusted by the addition of manganese sulphate.

The growth rate and feed efficiency were very satisfactory with no significant differences between the low-manganese and the high-manganese groups. During the 133-day growing period, *i.e.*, from 9.5 to 194.0 lb., the pigs on the low-manga-

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From the Department of Veterinary Science and Animal Husbandry (Plumlee), Purdue University, Lafayette, Ind.

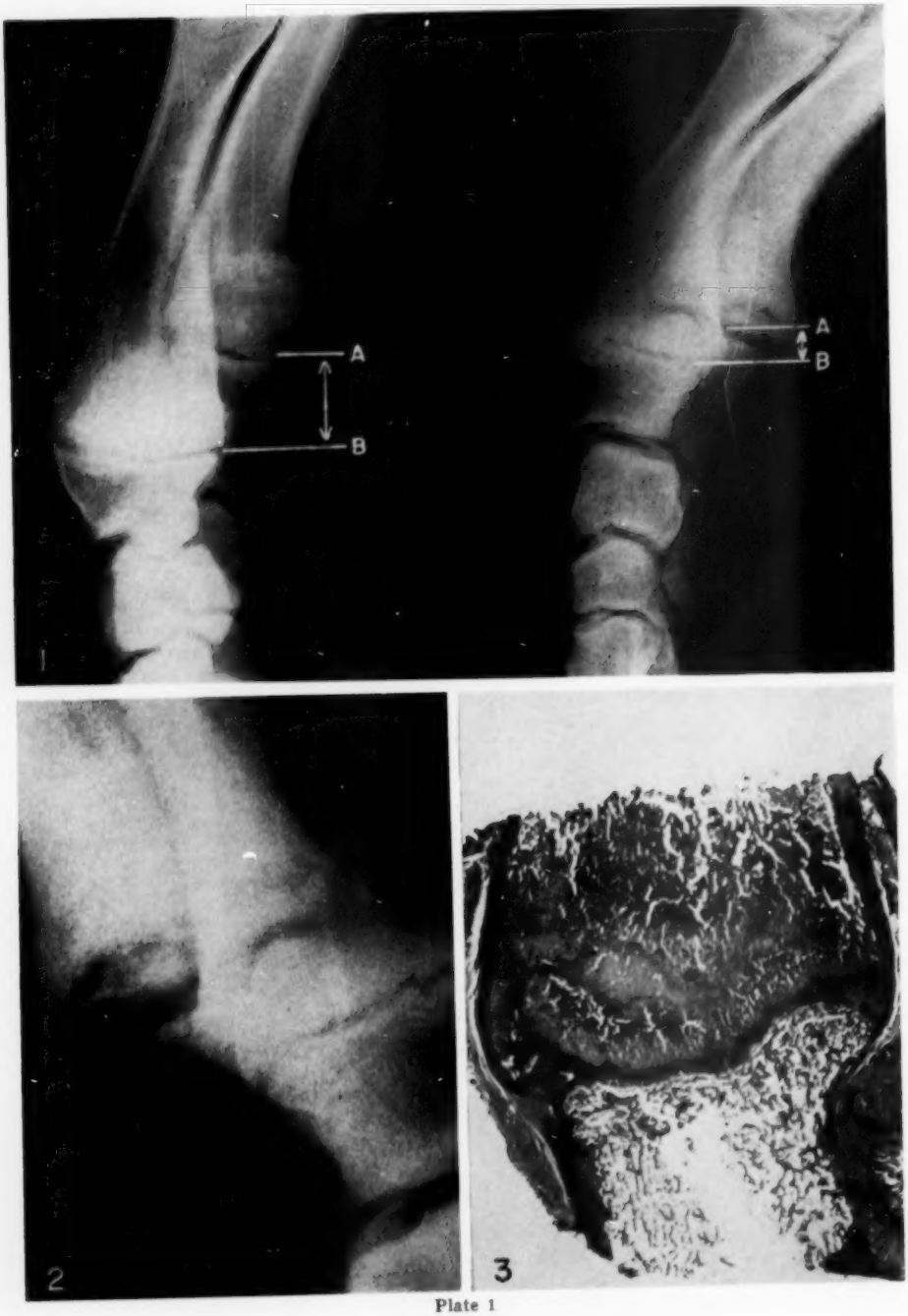

Plate 1

nese and the high-manganese rations had an average daily gain of 1.38 and 1.39 lb., respectively, and the feed efficiency was 3.13 and 3.06 lb. of feed per pound of weight gained. At the end of the growth period, *i.e.*, from the time the gilts weighed 200 lb., the fiber content of the ration was increased to 8 per cent and each animal received 5 lb. of feed daily, so that the growth rate was reduced to 1 lb. per day. They were maintained on this reduced feed intake until the experiment was terminated on the 335th day.

Radiographic examinations were made on 3 pigs deficient in manganese and on 1 control pig at the 110th day of the experiment, on 1 control and 1 deficient animal at the time of necropsy on the 125th day. Radiographs were also taken at the termination of the experiment and at necropsy (335 days) on 3 manganese-deficient and 1 control animal. Bone specimens were obtained at the time of slaughter, decalcified in an ionic decalcifier, and sectioned at 15 μ. The histological slides were stained by the usual hematoxylin and eosin procedure.

RESULTS

Clinical Findings.—Signs were first observed in the manganese-deficient animals at an average weight of 60 lb. The shoats showed weakness of the legs by remaining in a sitting position while eating; when standing they had a tendency to constantly shift weight on their hindlegs. These signs continued and when the average weight was 90 lb., they appeared shorter in length, thicker, and deeper bodied, and 6 of the 9 had a tendency toward bowed front legs. At 135 lb., the animals were less active and had a tendency to become excessively fat. The legs were markedly shorter and were thicker, particularly in the tarsal and carpal areas. The most striking sign was the bowing of the front legs. At 165 lb., the average thickness of the back fat of the pigs receiving the low-manganese ration, measured in three places, was 1.63 inches as compared to 1.40 inches for the control animals.

The deficient swine appeared more mature at 200 lb. than did those which received 40 p.p.m. of manganese in the ration. Although feed consumption remained fairly constant, most additional weight was manifested in the form of increased body fat. At this time, the lameness was not as apparent as it had been when the animals weighed 135 lb. As the experiment continued, no lameness attributable to manganese deficiency was observed in either group. However, the shortness of the legs, the enlargements of the carpal and tarsal areas, and the bowing of the front legs persisted.

Radiographic Findings.—Radiographic examination of 3 animals deficient in manganese and of 1 control animal on the 110th day and of 1 manganese-deficient animal and 1 control animal on the 125th day showed that a marked shortening of the radius, ulna, and metacarpal bones had occurred. The tibia, fibula, and metatarsals were also shortened as compared to the bones of the control animals. Medial radiographs demonstrated that an atypical relationship existed in the distance between the distal epiphyseal line of the radius and that of the ulna. This distance was markedly shorter in all manganese-deficient animals (fig. 1). The epiphyseal cartilages at the distal ends of the radius were consistently narrower and more serrated in appearance, indicating that closure of the growth discs was under way, whereas the width and appearance of the distal epiphyseal plates of the ulnas suggested that growth had continued.

In all manganese-deficient animals examined, the distal ends of the radius and ulna, at the epiphyseal lines, were markedly thickened so that the diaphysis appeared to be extended or "lipped" past the proximal edge of the adjacent epi-

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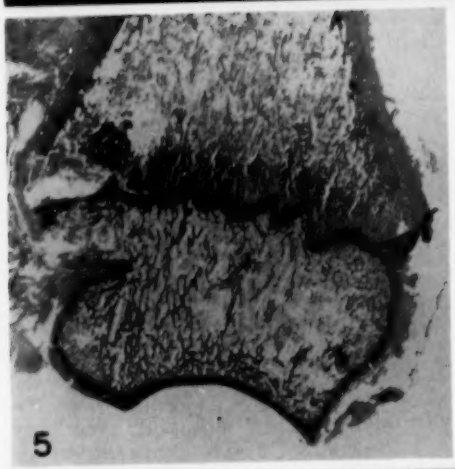
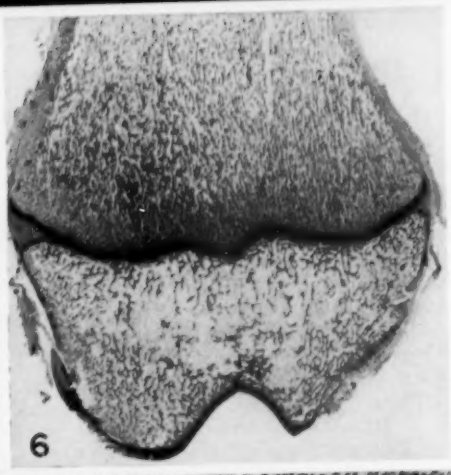
Plate 1

Fig. 1—Medial radiograph of the carpus of a control animal (right) and one after receiving a ration deficient in manganese for 125 days (left). A marked decrease in distance A-B between the epiphyseal growth discs of the distal radius and ulna occurred. In the deficient hog the growth plate of the radius appears thinner and serrated, indicating retarded osteogenesis. A detectable degree of rarefaction of bone is also evident in the deficient animal (left).

Fig. 2—An enlarged portion of a radiograph (125 days) of the distal ulna showing the typical rarefied lesion proximal to the growth plate and marginal lipping of the distal metaphysis over the adjacent epiphysis.

Fig. 3—Photograph of a histological section of the ulna showing that the corresponding rarefied lesion was occupied by a well-defined mass of fibrous tissue. x 4.





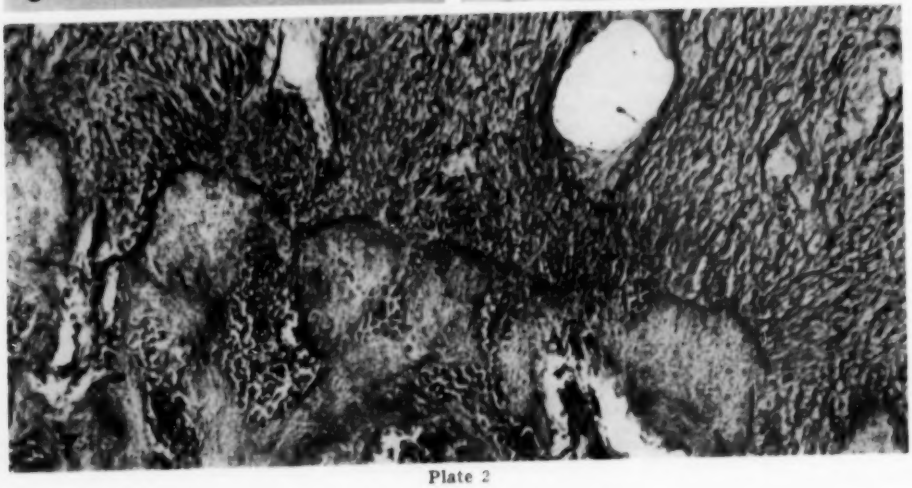


Plate 2

physis (fig. 1 and 2). This bony dystrophy was also apparent but to a lesser degree in the tarsal area.

Rarefied areas were observed in the distal diaphysis of the ulnas in the 4 animals deficient in manganese examined at 110 and 125 days. These lesions varied from 3 to 5 mm. in thickness and were located 1 cm. proximal to the growth discs (fig. 2). Figure 3 is an enlarged photograph of a histological section of the same portion of the ulna, and its pathology is discussed in a later section. Radiographic examination of 3 manganese-deficient and 1 control litter mate on the 335th day no longer showed any specific rarefied areas in the bones of the forelegs or hindlegs; however, a generalized rarefaction was evident in the deficient animals.

All of the leg bones of the manganese-deficient animals were shorter and thicker than those of the normal animal. The epiphyseal cartilage plates appeared to be almost completely closed. However, the atypical relationship of the distance between the growth plates, as previously mentioned, had persisted so that the appearance was similar to that observed at 110 days. The widening of the distal portion of the diaphysis of the radius and ulna and the general enlargement of the bones in this area (fig. 4), as well as in the tarsus, was more pronounced at 335 days.

Microscopic Findings.—Histological examination of the slides prepared from the animals slaughtered on the 125th day revealed that the distal cartilage discs of the radiuses were consistently thinner and more serrated in appearance in the manganese-deficient animals (fig. 5 and 6), whereas the width of the distal epiphyseal plates of the ulnas appeared to be normal. It would seem that manganese deficiency resulted in the selective closure of the growth discs of the radiuses; however, the same response was not elicited in the epiphyseal plates of the distal ends of the ulnas.

The most significant finding at 125 days in the radiuses of the animals deficient in manganese was a marked thinning of the epiphyseal cartilage. A disarrangement of the architecture of the proximal growth plates had occurred, so that some of the columnar arrangement of the cartilage cells was lost. There was an increase in the amount of matrix which was pale staining and located adjacent to the zone of proliferation. However, cytomorphologically, the cartilage cells appeared to be normal for swine of this age.

Microscopic examination of the areas in the diaphysis of the ulna which appeared rarefied in the radiographs (fig. 2) revealed an absence of cancellous bone. The normal bone trabeculations were either replaced or were aplastic. The rarefied spaces contained a dense growth of apparently old fibrous tissue which was moderately vascular. The peripheries of the fibrous lesions were clearly defined; however, some invasion of the adjacent marrow spaces was observed (fig. 7).

In the growing swine (110, 125 days), no increase in the amount of fat in the marrow spaces was observed in either the deficient or control shoats. However, in all mature swine which were fed the ration deficient in manganese, a marked increased deposition of fat was evident in the marrow spaces of both the epiphysis and diaphysis of the radiuses and ulnas. Since a prominent feature of this deficiency syndrome in swine is one of obesity, it would

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Plate 2

Fig. 4—Medial radiograph of the carpus of a hog raised on a manganese-deficient ration (335 days) showing a generalized rarefaction of the radius and marginal extension of bone over the adjacent epiphysis (C). The distal ends of the radius and ulna are enlarged.

Fig. 5—Photograph of a histological section of the distal end of the radius of a manganese-deficient hog (125 days) showing evidence of epiphyseal closure and generalized rarefaction of bone in the diaphysis. x 3.

Fig. 6—A corresponding section of the radius of a control animal which received adequate manganese showing a normal epiphyseal cartilage and the normal amount of cancellous bone. x 3.

Fig. 7—Photomicrograph of a portion of a fibrous mass which was present in the rarefied areas of the distal ulnas. The connective tissue was dense, apparently old in type, and moderately vascular. The periphery was clearly defined; however, invasion of the adjacent marrow spaces was evident. x 70.

appear that manganese may have a marked lipotropic action both for body fat and adipose tissue in bone.

DISCUSSION

The results of these experiments confirm the observations of previous reports in that lame pigs were produced when rations deficient in manganese were fed. However, lameness was apparent when the animals weighed 60 lb. rather than 150 lb. as reported by Miller *et al.*[5] The general syndrome, characterized by lameness, shortness of stature, enlargements of the carpus and tarsus, and a tendency toward bowing of the front legs, was similar to that previously reported.

It has been established that manganese deficiency is a common cause of perosis in poultry. The detailed studies of Wolbach and Hegsted [1] have suggested that the immediate cause of the deformities in chickens is a suppression of endochondral bone growth at the epiphyseal cartilages. Skeletal manifestations of a deficiency of this mineral have been observed in other species. Smith *et al.*[2] demonstrated that manganese deficiency in the rabbit was associated with retarded bone growth, deformities particularly of the front legs and decreased bone-breaking strength. Deficient rats showed no bone deformities *per se;* however, the offspring of deficient females and normal males had a high incidence of deformed tibias.[3] Shils and McCollum [4] reported similar findings in young mice except that the abnormalities were manifested chiefly as a bowing of the front legs. Amdur *et al.*[10] failed to observe deformities in this species but did report a marked decrease in breaking strength as well as some loss of bone density.

Radiographic and histological studies in this experiment showed that a slight generalized rarefaction of the bones of deficient swine had occurred (fig. 1, 5, and 6), and areas of complete rarefaction were observed in the distal diaphysis of the ulna of all deficient swine examined on days 110 and 125 (fig. 2). The examination of these lesions microscopically (fig. 3 and 7) revealed a loss of osseous tissue and a replacement with dense, apparently old, fibrous, connective tissue. Rarefied lesions were not observed either radiographically or histologically in the bones of deficient animals at 335 days (fig. 4). The disappearance of the rarefied areas coincided with the loss of lameness, so that the chief symptom present after the growth period was bone deformity without lameness.

A number of osteodystrophies described in man are characterized by rarefaction of osseous tissue and its replacement by young, actively growing, fibrous tissue. The pathological changes in the lesions observed in the swine ulnas are most comparable to the localized form of osteitis fibrosa (von Recklinghausen's disease). This dystrophy occurs at the end of one or more of the long bones during the growth period and spontaneous recovery frequently occurs later. The etiology is unknown. However, the immediate cause seems to be a malfunction of the normal process of the removal of calcified cartilage by vascular connective tissue which precedes true bone formation.

The authors have no good explanation for this apparently specific displacement or absence of bone from the rarefied areas. It would seem that atypical neo-osteoid extensions have arisen on the margins of the ulna immediately proximal to the growth discs, possibly at the expense of endochondral bone formation in the adjacent inner portion of the bone. Radiographically, these extensions of the diaphysis seem to form a ledge over the adjacent epiphysis (fig. 2 and 4).

It is of interest that the distal epiphyseal discs of the ulna of all manganese-deficient animals appeared to be open so that the rate of bone growth was more nearly normal, whereas the distal growth plates of the radiuses were consistently thinner and more serrated in appearance as compared to the control animals. These observations suggested that in pigs deficient in manganese, the ulnas continued to grow normally for a time while growth of the radiuses was retarded. The results of this atypical growth pattern were manifested in the radiographs and histological sections by a marked shortening of the distance between the growth discs of the ulna and radius at 125 days (fig. 1). The later radiographs (335 days) indicated that closure of the growth discs of the ulna had occurred; however, the atypical arrangement had persisted (fig. 4). Without doubt the abnormal growth relationship of these

bones could account for the marked bowing of the front legs of the manganese-deficient swine.

The lipotropic action of manganese has been demonstrated in the rat.[13] Deficient animals developed fatty livers which were prevented by the addition of manganese to the ration. Norris and Heuser[14] further showed that an increase in fat in the bone accompanies the deficiency in the rat. In all mature swine deficient in manganese (335 days), a marked increase in fat cells occurred in the marrow spaces of both the diaphysis and epiphysis of the radiuses and ulnas. This finding was not surprising since one of the prominent features of this deficiency syndrome in swine was excessive obesity.

The role of manganese in bone formation is not clearly understood; however, there is evidence to suggest that its action may be mediated through the enzyme phosphatase. Manganese deficiency occurs concomitantly with low phosphatase activity of the blood and bone in poultry[11,12] and in the rat.[13] Phosphatase is believed to be produced by proliferating cartilage cells and osteoblasts. It liberates inorganic phosphate from phosphoric esters and raises, locally, the concentration of phosphate ions so that the levels of both calcium and phosphate ions increase, and the deposition of calcium phosphate in the cartilagenous matrix occurs.

The fact that phosphatase activity is lowered in the bones of the manganese-deficient rat and the manganese content of the bone remains within the range for normal rats suggests that a discrepancy may exist in the supposition that phosphatase activity is dependent upon normal concentrations of manganese in the bone. In deficient swine showing bony dystrophies of the legs, no decrease in the manganese content of the bones was evident.[5,6] Plumlee and his co-workers[15] reported that although the manganese content of bones from deficient swine was normal, there was a threefold to fivefold decrease of this mineral in soft tissues. This suggests the possibility that total stores of the mineral in the body may be concerned with the phosphatase activity in osteogenesis and not the absolute amount present in the bones *per se*. Studies designed to measure the dynamics of manganese utilization by normal bone in contrast to that of deficient swine may help to answer this question.

Summary

1) Manganese deficiency in swine resulted in lameness, shortness of bone length in the forelegs and hindlegs, a thickening of bone in the carpal and tarsal areas, and a marked bowing of the front legs in 60 per cent of the animals. After maturation the osteodeformities remained; however, the lameness had disappeared.

2) Radiographic and histological examination revealed a selective retardation of endochondral osteogenesis in the growth discs of the radiuses, whereas the ulnas continued to grow so that bowed front legs resulted.

3) A detectable, generalized rarefaction of bone occurred and areas of complete aplasia were observed in the distal diaphysis of the ulnas of all deficient animals examined at 110 and 125 days. At 335 days, no similar rarefied areas were present.

4) These lesions appeared similar to the localized form of osteitis fibrosa and were characterized by the replacement of cancellous bone with a dense fibrous tissue which was moderately vascular. The disappearance of these areas occurred concomitantly with the recovery from lameness.

5) Neo-osteoid extensions proximal to the growth discs and adjacent to the aplastic lesions were a typical finding.

6) The lipotropic action of manganese was demonstrated by the excessive obesity of the deficient swine as well as an increased deposition of fat in the bone.

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Practical Isolation Equipment for Baby Pigs

E. O. HAELTERMAN, D.V.M.

Lafayette, Indiana

ISOLATION of animals is a necessity in many phases of research with infectious diseases where it is essential that the exposure of test animals be rigidly controlled. Equipment that will fulfill the requirements for most infectious disease work should have the following characteristics: (1) a system of ventilation which will prevent air-borne infection between units or from outside sources, (2) a means of feeding animals which will minimize the danger of cross contamination during these operations, (3) adequate storage capacity for excreta so that the unit will not have to be opened during the animal's stay in the unit, (4) provision for adequate visual observation, (5) construction should be in a manner and from materials that will permit easy and efficient cleaning and sterilization, and (6) the cost of the equipment should be low enough to permit its use in laboratories with limited budgets.

EQUIPMENT

Several types of small animal isolation equipment have been described. Horsfall and Bauer[1] described isolation equipment which they used in the study of distemper and influenza in ferrets. Their units consisted of monel metal cubicles in which a small animal cage was placed. Ventilation was accomplished by a vacuum system which drew air from the room into the units and exhausted it outside. Air filters were provided over the openings through which air entered and left the cubicles. Young and Underdahl[2] developed similar isolation equipment which was modified for feeding and maintaining pigs. These units were used successfully in growing pigs without colostrum and also in the study of transmissible gastroenteritis and other virus diseases in pigs. Levine and Fabricant[3] described isolation cages which were used in the study of Newcastle disease in chicks. These units consisted of galvanized iron cubicles containing cages for birds. This equipment differed essentially from the first two described in that ventilation was accomplished by a system which was closed both to and from the units. The air within the cubicles was maintained at a slight positive pressure by means of a fan which forced outside air through ductwork into the cubicles and returned it to the outside.

The isolation equipment described in this paper was designed for and has been used successfully in the study of transmissible gastroenteritis (TGE) in pigs. Susceptible pigs have been maintained for periods up to three weeks while pigs affected with TGE were being kept in adjacent units. The equipment has also been used successfully in raising pigs deprived of colostrum. With appropriate modifications in size and feeding facilities, this equipment might be adapted for use with other animals.

The bodies of these units were made from Witt No. 3* plain bodied galvanized cans. These cans are 20 inches in diameter and 26½ inches high. Each can was fitted with an expanded metal platform which was set on brackets placed 5 inches above the floor (A, fig. 1). A feed pan (B) was placed in a framed opening in the side of the can and secured with a metal latch (A, fig. 2). One end of the latch was slotted to permit removal of the feed pan without removal of the wing nuts. A rubber gasket was used under the flange of the pan to provide an air seal. A half-inch copper tube (C, fig. 1 and 2) through which liquids could

* Witt Cornice Company, Cincinnati, Ohio.

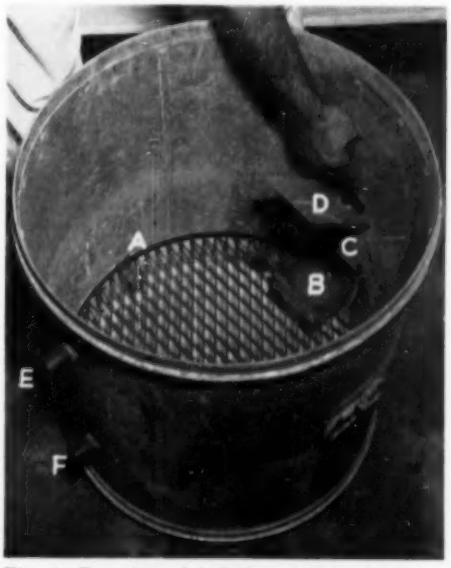

Fig. 1—Top view of isolation unit showing false floor (A), feed pan (B,C), and cover (D). The upper nipple (E) is for the air inlet and the lower (F) for air outlet.

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Fig. 2—Front view of isolation unit showing feeding tube (C) and mechanism (A) for holding feed receptacle (B).

be poured for feeding was fitted into the side of the can just above the feed pan. The external end of this tube was closed with a rubber stopper. A hinged lid (D, fig. 1) was attached over the feed pan to prevent soiling of the feed by the animal. The top of the unit was covered with heavy gauge (du Pont, 600 MST) cellophane which was secured with a rubber band cut from the inner circumference of a 15-inch automobile inner tube.

The ventilating system is somewhat similar to the one devised by Levine and Fabricant. A positive air pressure within the units was provided by a centrifugal fan** (A, fig. 3) connected to a manifold and ducts to draw air from outside through a chimney. The air intake duct (C) was made of 4-inch galvanized tubing and was provided with a fiber glass filter. This was the only filter in the system. The manifold (B) was divided by an airtight partition into upper and lower compartments each of which was fitted with eight nipples to which lengths of soft rubber tubing (1 in. dia.) were attached to conduct air to and from the units. The exhaust duct (D) was made of 6-inch galvanized tubing. The positive pressure within the units prevents the entrance of contaminated air during feeding.

OPERATION

Most of the pigs used for work with TGE were from 3 to 5 days old when removed from the sow

** Fasco No. 50749-1N, 115 volt, 1.25 amp., Fasco Industries, Rochester, N. Y.

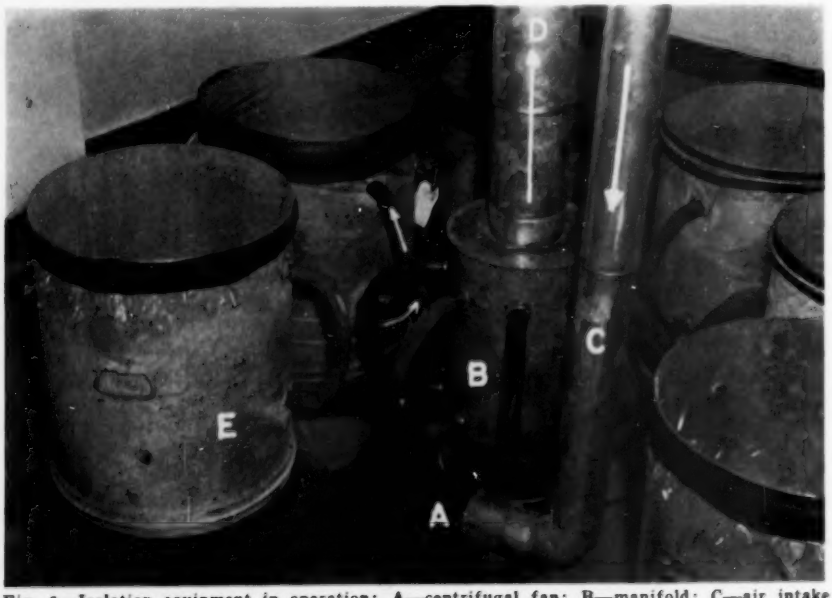

Fig. 3—Isolation equipment in operation; A—centrifugal fan; B—manifold; C—air intake duct; D—exhaust duct. Arrows indicate direction of air flow. Unit E is sealed for removal from the room.

and placed in the units. The feed for these animals consisted of homogenized, pasteurized cow's milk containing 3 cc. of 5 per cent ferrous sulfate per quart. Milk was poured directly from the delivery bottle into the feeding tube. Pigs taken from the sow rarely take more than a few hours to learn to eat from the pan and none has been lost because of failure to learn to eat since this type of unit has been used. If the amount of feed was regulated so that it would be consumed before the next feeding, it was not found necessary to clean the feed pans oftener than once every two or three days.

Pigs deprived of colostrum were fed a diet formulated by Young *et al.*[4] containing one whole egg per quart of milk and a mineral mixture. For the first two days of the pig's confinement this formula was boiled before being fed. Also the feed pans were removed after every feeding and replaced with clean pans which had been sterilized by boiling. All pigs were fed three times daily until 1 week of age and twice daily thereafter.

Pigs may be kept in these units up to 3 or 4 weeks of age, the size of the animal being the limiting factor. The space beneath the false floor is large enough so that it does not have to be cleaned during the animal's stay.

When an infected animal is to be removed from a unit, the rubber air intake tube is disconnected from the unit and bent upward and connected to the upper compartment of the manifold. The exhaust tube is also bent upward and connected to the intake nipple of the unit. The entire unit, thus sealed to prevent contamination of the isolation room, is carried outside of the room before it is opened. The air intake tube shunts the air, which would otherwise flow through the unit, to the upper compartment, thus preventing an excess of air from flowing through the remaining units. Figure 3 shows a unit (E) ready for removal from the room.

Cleaning and sterilizing of the units is accomplished by rinsing out the accumulation of excreta and boiling in a large tank of water containing caustic cleaning compound, or by merely filling the unit with water after closing the feed tube and connecting the air exhaust and intake nipples with an air tube and boiling with steam. The entire room with the equipment is fumigated with formaldehyde before the start of each experiment.

The cost of this equipment for materials and labor was less than $40 per unit including the ventilating system which services eight units.

Summary

Inexpensive animal isolation equipment which has been used successfully in the study of transmissible gastroenteritis in pigs and in raising pigs deprived of colostrum is described. The units afford good visibility of test animals, a convenient means of feeding with minimal danger of cross infection and are easily cleaned and sterilized.

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Laboratory Studies on Erysipelas. II. Use of Various Cultures in Production of Infection in Pigs by Skin Scarification

H. E. GOUGE, D.V.M.; R. BOLTON, B.S.; R. BROWN, D.V.M.

Pearl River, New York

INCONSISTENCY of experimental reproduction of swine erysipelas is recognized as probably the most serious drawback encountered in the study of any type of immunizing agent against this disease.

On several occasions, the disease has been reproduced by oral, intravenous, intramuscular, subcutaneous, or intraperitoneal injection of culture,[1-3] but such results are usually not obtained with any degree of regularity. Similar experiences have been reported following application of the scarification method of Fortner and Dinter.[4] In addition to some foreign workers, Shuman,[5] Cooper and his associates,[6] and Spencer[7] have successfully used the scarification method, while Doyle,[8] among others, has reported difficulties. Various explanations have been offered for these discrepancies reported, including differences in strains of the organism, differences in animals, and absence of possible predisposing factors.[7, 9]

Studies were undertaken to attempt to establish a suitable erysipelas challenge which could be reproduced routinely, and the present report is the outcome of this work. Cultures were obtained from other investigators interested in and working with the problem in the United States and in Europe. These were administered intravenously, intradermally, intraperitoneally, subcutaneously, intraocularly, intranasally, and by scarification to pigs from various sources and of different ages. Cultures of varying concentrations and dilutions were used and, in some cases, were combined with various accessory activating agents. From the results obtained in these tests, it was concluded that, if challenge results were to be measured in terms of the development of acute disease and death, none of the procedures used proved satisfactory. However, quite consistent cutaneous, systemic, and febrile reactions were obtained with the skin scarification technique, not only by us but also by others.[5, 7] Although, in general, the intravenous method had appeared to be a somewhat more severe challenge, as was pointed out by Sikes, Neher, and Doyle,[10] it was expensive because of mortality of test animals, and it did not lend itself to readily measurable results in comparative laboratory studies, whereas scarification did. It was concluded, therefore, that the characteristic febrile, systemic, and skin reactions of the scarification method could be used as criteria in future studies.

From the Research Division, Lederle Laboratories American Cyanamid Co., Pearl River, N. Y.

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This is a report of further studies on these reactions obtained with cultures from different sources to determine which would produce the maximum response. With possible standardization of challenge methods in mind, Shuman's cultures[11] were included, and results were interpreted in terms of Shuman's classification of reactions.[11]

MATERIALS AND METHODS

Challenge Cultures.—The following cultures of *Erysipelothrix rhusiopathiae* were used:

1) Shuman—received from Dr. R. D. Shuman, Beltsville, Md.; 4 cultures, S192, HC585, 422-1, NF4 (11); experimental strains; lyophilized preparations;

2) German—received from Dr. K. L. Wolters, Germany; 3 cultures, 846, 847, 848, routinely used for swine scarification; lyophilized preparation;

3) Field—received from Dr. George Mueller, St. Louis, Mo.; field strains; tissues, frozen;

4) Canadian—received from Dr. H. C. Rowsell, Department of Bacteriology, Ontario Veterinary College, Guelph, Canada; experimental strain; lyophilized preparation;

5) Purdue—received from Dr. Dennis Sikes, Purdue University; experimental strain; lyophilized preparation.

Before use, all strains were passed through mice, and then were cultivated at 37 C. for 18 hours in yeast bile broth containing 10 per cent horse serum.

In the early studies, the German cultures (No. 2) were used. It was decided to investigate the possibility of obtaining a culture of greater virulence. Tissues were obtained from 2 pigs dead of erysipelas from a severely affected herd. The organisms were recovered from these tissues on blood agar plates, stored on serum agar plates, and subcultured periodically (culture 3).

Animals.—Apparently normal pigs, approximately 3 to 4 months old and susceptible to swine erysipelas, were used. The pigs were identified by ear tags, were housed in concrete pens, and were fed a regular commercial ration not supplemented with antibiotics.

EXPERIMENTAL DATA

Twenty-five pigs were divided into five equal groups, one group for each of the five cultures tested. All temperatures were normal before scarification. Temperatures were recorded, pigs were observed for incidence of clinical disease, and scarification sites were examined for extent of erythema daily for eight days following scarifica-

tion. Shuman's classification[5] of the results of scarification, adopted in an attempt to standardize so far as is possible the interpretation of results, is as follows: Immunity = absence of local reaction; incomplete immunity = transient and slight local reaction; susceptibility, localized = persisting local reaction; susceptibility, generalized = persisting reaction becoming generalized. If any of these reactions was obtained at any scarification site, not necessarily at all sites, the reaction was recorded. Final classification of the animal was made on the basis of the maximum reaction.

RESULTS

Clinical symptoms were most pronounced in pigs inoculated with the Shuman strain. Inactivity and inappetence or reduced appetite were evident in 2 of the 5 pigs for the entire eight-day observation, and in 2 others for six days. Labored breathing was noted in 2 pigs. With the Purdue and field strains, only 1 animal in each group was inactive, each refusing food for eight days. Inactivity and inappetence were evident in the others in these groups on the fourth day, and persisted for two or three days, while respiration remained normal. In pigs inoculated with the Canadian and German strains, inactivity and reduced appetite were evident only on the fourth day, with one exception, and respiration remained normal.

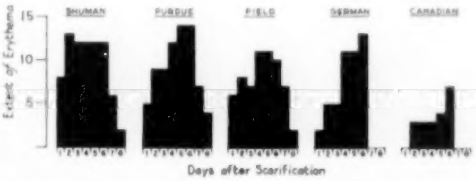
Average temperatures are shown (graph 1). Pigs inoculated with the Shuman strain showed the two-phase temperature reaction reported by Shuman.[5] The Purdue strain roughly paralleled the Shuman strain, as did the field strain to a slightly lesser degree. The febrile reaction following administration of the German and Canadian strains was much less pronounced.

The extent and duration of cutaneous reactions closely paralleled the febrile reactions (graph 2) with the most severe and peristent erythema occurring in those groups showing the highest and most persistent temperatures. To plot graph 2, each classification was given a numerical value from "0" for no reaction through "3" for generalized reaction and total reaction for group recorded.

Therefore, since the Shuman strain of *Ery. rhusiopathiae* gave the maximum reactions, and since it offered the possibility for standardization of interpretation of results of experiments by different investigators, this strain was chosen to serve as challenge culture in future studies.

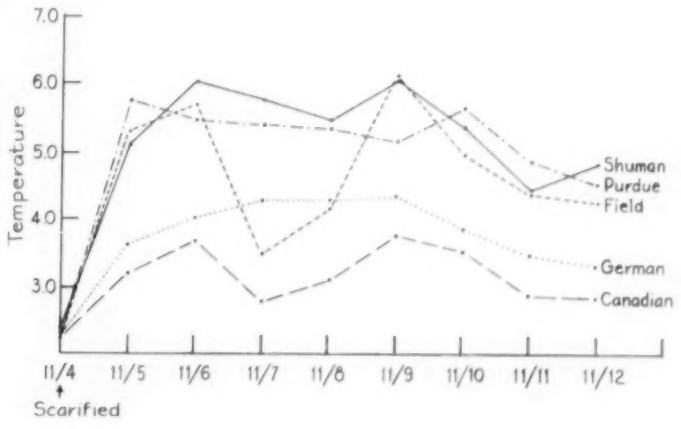
DISCUSSION

It would appear that a better understanding of swine erysipelas and evaluation of immunizing agents could be accom-



Graph 2—Comparative degree of skin erythema following scarification (5 animals per culture).

Graph 1—Average (5 pigs per culture) temperatures following scarification.



plished if the method of challenge, classification of reactions, and challenge culture could be standardized. Quite consistent cutaneous, systemic, and febrile reactions have been obtained with the skin scarification technique. Systemic and febrile reactions, in the experiment reported, were similar to those reported by Shuman,[11] and the Shuman classification of cutaneous reactions was readily applicable. In addition, the Shuman culture strains produced the maximum reactions. It would appear, therefore, that consistent results, sufficiently typical to be used as a means of interpretation, can be obtained under various experimental conditions.

SUMMARY

Erysipelothrix rhusiopathiae cultures from five sources were administered to susceptible pigs by skin scarification. Clinical symptoms, febrile and cutaneous reactions were recorded. The Shuman strains of cultures produced the maximum reactions and the Shuman classification method was readily applied.

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Laboratory Studies on Erysipelas. III. Duration of Immunity in Pigs Vaccinated with Adsorbed Bacterin, and with Serum and Culture

H. E. GOUGE, D.V.M.; R. BOLTON, B.S.; M. C. ALSON, D.V.M.

Pearl River, New York, and South Saint Joseph, Missouri

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Adsorbed swine erysipelas bacterin has been widely field tested and routinely used in Europe since its introduction in 1947 by Traub[1] and Dinter.[2] Judged by clinical use in areas where swine erysipelas had recurred year after year, immunity proved satisfactory for four to six months.[3-6] In the United States, similar bacterins have been in use for only a short time, and a single report has appeared on routine clinical use. Callaway and his associates,[6] reporting on more than 9,000 pigs, stated that, for the eight months from October, 1953, to May, 1954, pigs vaccinated with this type of bacterin were raised and marketed without complications from erysipelas such as had ordinarily been experienced in previous years on the same premises. The majority of the pigs were vaccinated before or shortly after weaning, but pigs from 3 to 12 months of age were included. Animals were observed to 6 months of age for market pigs, and for 6 months postvaccination for breeding stock.

However, experimental laboratory studies reported by Shuman[7] showed less encouraging results when the scarification method of challenge was used following vaccination with one 5-cc. dose of bacterin. One experimental bacterin was administered as a single dose to 36 pigs, groups of which were challenged at monthly intervals for five and one-half months, together with a total of 15 unvaccinated controls. A second bacterin was given as a single dose to 19 pigs, with challenges conducted monthly for three months, using 8 unvaccinated controls. In the first test, all pigs challenged were resistant one month following vaccination. The degree of protection dropped rapidly thereafter and at five and five and one-half months, no protection was demonstrated. All controls proved susceptible. With the second bacterin, only 33⅓ per cent protection was evident at one month, 11 and 25 per cent at 2 and 3 months, respectively, and 75 per cent of the controls were susceptible. However, only 6 of the 41 vaccinated pigs classified as susceptible in the two groups developed generalized infection. Comparing the results of this test with tests with serum and culture previously reported,[8] Shuman concluded[9] that the latter method appeared more satisfactory on the basis of experimental challenge with cultures of known infectivity.

As the best basis for comparison of two methods of vaccination is to conduct simultaneous studies, experiments were designed to determine duration of immunity conferred on pigs by inoculation with an adsorbed erysipelas bacterin, and with serum and culture, using the skin scarification method of challenge.

Material and Methods

Immunizing Agents.—The erysipelas bacterin* was a commercial lot prepared from chemically killed, whole broth cultures of three highly antigenic strains of *Erysipelothrix rhusiopathiae*, precipitated with aluminum hydroxide, concentrated, and potency-tested in mice.[10]

Commercially produced anti-swine erysipelas serum and desiccated erysipelas vaccine were used for serum-and-culture vaccination. A report from the laboratory of origin indicated that, at the time of production, the vaccine killed 2 mice within three days and the third mouse within five days. This is in accordance with the test for acceptable vaccine which requires that all of 3 mice should die within seven days after infection with 0.1 cc. of the reconstituted vaccine inoculated subcutaneously. Retest of the culture vaccine at time of use corroborated this report.

Challenge Culture.—Cultures of *Ery. rhusiopathiae* from five different sources were tested in pigs by the scarification method to obtain as highly virulent a strain as possible.[10] The four strains used by Shuman[7] and obtained from him, identified as 8192, HC585, 422-1, and NF4, were chosen. Each of these four strains was tested separately, but no significant differences in virulence could be detected. Therefore, to insure as severe a challenge as possible, the four strains were combined after being grown for 20 hours at 37 C. in yeast bile broth containing 10 per cent horse serum.

Animals.—Mixed breed, average market-type pigs, approximately 8 weeks of age, were obtained from two farms where erysipelas had never occurred as far as could be determined. The pigs were housed by groups in pens with concrete floors and were fed a commercial growing ration

From the Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y. (Gouge and Bolton) and South St. Joseph, Mo. (Alson).

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*Duovax® erysipelas bacterin, Lederle Laboratories.

TABLE 1—Design for Erysipelas Immunizing Experiment

Group	No. pigs	Immunizing agent*	Dose	Date of vaccination		Number pigs challenged on		
				1st	2nd	1/7/55	2/8/55	3/10/55
1	60	Bacterin 1 dose	5.0 cc.	11/9/54	.....	20	20	20
2	38	Bacterin 2 doses	5.0 cc.	11/9/54	12/6/54	...	20	18
3	56	Serum and culture	10.0 cc.	11/9/54	.....	20	18	18
4	63	Unvaccinated controls	0.5 cc.	.....	.....	20	20	23

* Subcutaneous administration.

not supplemented with antibiotics. Pens were cleaned at least once daily throughout the trial. The pigs were 2½ months old at the time of erysipelas vaccination.

Procedure.—The 223 pigs were penned and vaccinated against hog cholera 14 days before the start of the trial. Six pigs died of intercurrent infection in the two-week holding period. The remaining 217 were divided into four groups for an immunizing experiment (table 1). Erysipelas bacterin was administered subcutaneously in the flank in a single dose of 5 cc. to 60 pigs in group 1, and two 5-cc. doses at an interval of approximately one month to the 38 pigs in group 2. The 56 pigs in group 3 were vaccinated subcutaneously with 0.5 cc. of living culture vaccine and 10 cc. of anti-erysipelas serum, as recommended by the producer. The 63 pigs in group 4 were held as unvaccinated controls.

As Cooper and his associates[11] had found that a single 5-cc. dose of bacterin apparently protected all of 31 pigs against challenge three weeks postvaccination, challenge in the present trial was not started until approximately two months following vaccination in all groups. The skin scarification inoculation method of challenge of Fortner and Dinter[12] was chosen following a comparison of results of several methods. From 18 to 23 animals from each group were challenged at monthly intervals, through the fourth postvaccination month for groups 1, 3, and 4, and through the third month for group 2 (table 1). Each pig was trimmed with an electric clipper on the side from the shoulder to the hip and four regular scarifications, just sufficiently deep to produce a slight serum ooze tinged with blood, were made at regular intervals with the tip of a scalpel which had been bent and roughened. Challenge culture was applied to the first three scarifications with a pullorum dropper and rubbed vigorously with a forceps. The fourth scarification served as control.

Temperatures were taken and pigs were individually observed at least once daily for nine days after challenge. They were observed as a group for 30 days. Febrile reaction as described by Shuman[5] and his classification of cutaneous reactions were used as follows: immunity = absence of local reaction; incomplete immunity = transient and slight local reaction; susceptibility, localized = persisting local reaction; susceptibility, generalized = persisting reaction becoming generalized. If any local reaction appeared, no matter how slight, the animal was classified as incompletely immune.

An added criterion was "stiffness," a term used to designate a sign of erysipelas characterized by the animals squealing when forced to move and by walking on their toes. In some cases, swollen joints were observed. The onset was usually noticed within 24 to 48 hours after challenge.

RESULTS AND DISCUSSION

The purpose of this study was to determine the value of immunizing agents in protecting pigs to market age. Pigs were approximately 2½ months old at the start of the trial; the first challenge was made

TABLE 2—Results of Challenge Following Immunization with Adsorbed Erysipelas Bacterin and Serum and Culture

Group	Immunizing agent*	Challenge at**											
		4½ months of age (1/7/55)				5½ months of age (2/8/55)				6½ months of age (3/10/55)			
		Immune	Incomplete immunity	Susceptible		Immune	Incomplete immunity	Susceptible		Immune	Incomplete immunity	Susceptible	
				Local	General			Local	General			Local	General
1	Bacterin 1 dose	17/20†	1/20	1/20	1/20	11/20	2/20	3/20	4/20	5/20	1/20	4/20	10/20
2	Bacterin 2 doses	.....	.....	.....	.....	15/20	3/20	1/20	1/20	13/18	0/18	3/18	2/18
3	Serum and culture	6/20	2/20	5/20	7/20	1/18	1/18	0/18	16/18‡	2/18	3/18	3/18	10/18
Control		2/20	1/20	2/20	15/20	1/20	1/20	1/20	17/20	3/23	1/23	2/23	17/23

* Vaccinated at 2½ months of age; ** challenge started two months after vaccination; † no. of pigs in immunity classification/total challenged; ‡ 1 died.

approximately two months following vaccination (tables 1 and 2). The pigs in all groups, except group 2 which received 2 doses of bacterin, were 4½ months of age at first challenge; three challenges were made at monthly intervals. Those in group 2 were 5½ months of age at first challenge and only two challenges were carried out. Results were analyzed statistically.

TABLE 3—Average Duration (Days) of Skin Reaction in Susceptible Pigs

Group	Time of challenge after first vaccination *		
	2 mo.	3 mo.	4 mo.
Bacterin, 1 dose	2.5 (2)**	1.7 (7)	3.1 (13)
Bacterin, 2 doses	....	1.5 (2)	2.2 (5)
Serum and culture	3.5 (12)	3.3 (16)	3.7 (13)
Control	3.5 (17)	3.7 (18)	3.5 (19)

* Vaccinated at 2½ months of age.
** ( ) Number of pigs in average.

Cutaneous Reactions.—In table 2, the numerator gives the number of pigs in the various reaction classifications and the denominator gives the total challenged, using cutaneous reaction as the basis of classification. For purposes of comparison by the *chi*-square method, the incompletely immune pigs were classified as immune, and both classes of susceptible pigs were considered susceptible.

At the two-month challenge, the immunity in the serum and culture group did not differ significantly from the controls, whereas one dose of bacterin conferred immunity significantly better than either. At the three-month challenge, immunity from serum and culture was again no better than in the controls, while in both the one-dose and two-dose bacterin groups it was significantly better, although the latter two did not differ significantly from each other. At the four-month challenge, neither the serum-and-culture group nor the one-dose bacterin group showed better immunity than controls, but in the two-dose bacterin group immunity was significantly better than in the other three groups. The use of the second dose would appear to prolong the antigenic activity and the resultant immunity. These results, therefore, indicate the added value of the second immunizing dose in the protection of pigs to market age.

The average duration of skin reaction in susceptible pigs (table 3) following challenge of pigs vaccinated with serum and culture, was essentially the same as the controls. In pigs vaccinated with one dose of bacterin, the reaction duration was one day less at the two-month and two days less at the three-month challenge than in the controls, while at the four-month challenge the duration of reactions was almost equal. Vaccination with two doses of bacterin produced results essentially the same as one dose at the three-month challenge, but at the four-month challenge (6½ months of age) duration of reaction was 1.3 days less than controls. Although these results indicate a trend, analysis by means of the "t" test showed a significant difference only between pigs vaccinated with one dose of bacterin and controls at the three-month challenge.

Temperature Deviation.—Temperatures were recorded on the day of challenge and for eight succeeding days. The number of pigs with a temperature rise of 2 F. or more above initial temperatures are shown (table 4) and the average deviation from the initial temperature is recorded (graph 1). In general, results paralleled observations on occurrence and duration of skin reactions and indicated the superiority of two doses of bacterin.

Statistically, fever curves were compared after each challenge for difference in average temperature elevation and rate of increase and decrease in temperature. Details will appear in another publication.[13] At the two-month challenge, temperature elevation in pigs vaccinated with serum and culture was significantly less than the controls, but it rose substantially as rapidly in both groups. In pigs vaccinated with one dose of bacterin, temperatures were very significantly lower than the other two groups and did not rise as rapidly.

At the three-month challenge, average elevation of temperature of pigs vacci-

TABLE 4—Temperature Rise, Following Challenge, of 2 F. or More Above Initial Temperature

Group	Time of challenge after first vaccination *		
	2 mo.	3 mo.	4 mo.
Bacterin, 1 dose	7/20**	12/20	14/20
Bacterin, 2 doses	....	4/20	3/18
Serum and culture	9/20	16/18	13/18
Control	18/20	18/20	13/23

* Vaccinated at 2½ months of age.
** No. pigs/total.

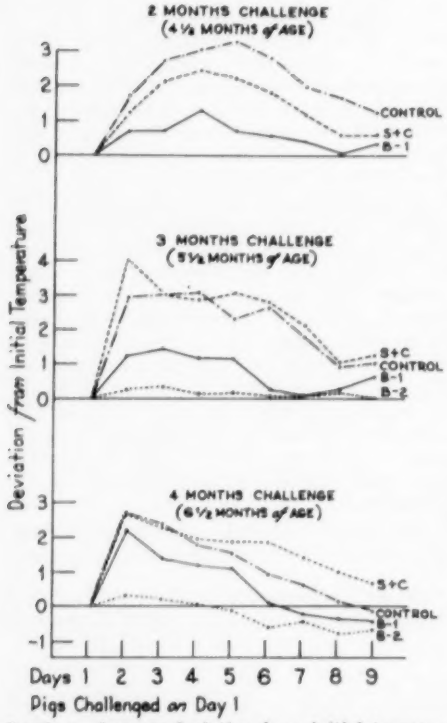
TABLE 5—Pigs Showing Stiffness (One Day or More) and Its Average Duration

	Time of challenge after first vaccination					
	2 mo.		3 mo.		4 mo.	
Group	No. stiff/total	Days (ave.)	No. stiff/total	Days (ave.)	No. stiff/total	Days (ave.)
1**	3/20	1.5	0/20	.....	6/20	1.0
2†	.....	.....	1/20	2.0	0/18	.....
3‡	9/30	2.9	17/18	4.6	14/18	1.1
Control	20/20	4.4	18/20	4.3	20/23	1.2

** Bacterin, 1 dose; † bacterin, 2 doses; ‡ serum and culture.

nated with one dose of bacterin was very significantly lower than the serum-and-culture and control groups. In pigs vaccinated with two doses of bacterin, the variation was not distinguishable from what might be expected in day-to-day temperatures of normal pigs.

At the four-month challenge, average elevation of the serum-and-culture group was higher than the controls but not significantly so. The average temperature elevation of the group vaccinated with one dose of bacterin was not significantly less than the control, but was very significantly less than the serum-and-culture group, while the deviation in those vaccinated with two doses of bacterin was very significantly less than the other three. In this group, there was a moderate drop in temperature which was significant, although unexplained.



Graph 1—Average deviation from initial temperatures following erysipelas challenge of pigs given one dose of bacterin (B-1), two doses of bacterin (B-2), serum and culture (S-C) in comparison with the controls.

Stiffness.—The number of pigs which showed stiffness for one day or more, and its average duration in those affected (table 5) quite closely followed the observations on cutaneous reactions and temperature. At the two- and three-month challenges following administration of one dose of bacterin, few or no pigs were affected, while at four months, the number had increased slightly. At the two-month challenge, the serum-and-culture group showed considerably less stiffness than the controls, but at three and four months, almost identical results were obtained. As in the other tests, the number reacting to challenge clearly indicated the value of the second dose of bacterin. Duration of stiffness was affected in a like manner.

Results were statistically analyzed by the use of the *chi*-square test for incidence and the "t" test for duration. At the two-month challenge, the incidence and duration of stiffness in both the serum-and-culture and the one-dose bacterin groups were very significantly less than the controls. They did not differ significantly from each other regarding incidence, but the duration of stiffness was significantly less in the one-dose bacterin group than in the serum-and-culture group.

At the three-month challenge, the serum-and-culture group no longer differed from controls in regard to either incidence or duration of stiffness. Those given either one or two doses of bacterin, on the other hand, had an incidence of stiffness which was very significantly less than either the

controls or the serum-and-culture group. The duration of stiffness in the two-dose bacterin group also was very significantly less, but as there was no stiffness in the one-dose group the duration could not be investigated.

At the four-month challenge, the serum-and-culture group was again no better than the controls, concerning incidence and duration. The two-dose bacterin group was significantly better than the one-dose group, and both were very significantly better than either control or serum-and-culture groups in regard to incidence. Duration of stiffness was greatly reduced in all groups at this time, which may be associated with age.

COMMENTS

Because of variability in immune response following vaccination with serum and culture in the dosages currently recommended, some investigators contend that the ratio between serum and culture should be more nearly balanced. In a controlled experiment, Shuman[14] demonstrated superior duration of immunity when weanlings were vaccinated with 0.5 cc. of culture and 3.0 cc. of serum, a ratio of 1:6, rather than the recommended ratio of 1:20. There is the possibility, therefore, that results of serum and culture administration in the present trials might have been more satisfactory had different dosage schedules been used. It must be pointed out, however, as Shuman has stated,[14] that until wide field use proves the safety of dosage changes, such recommendations can not be made. Thus, the currently recommended dosages were used in this experiment.

SUMMARY

A total of 154 weanling pigs were vaccinated against erysipelas either with culture and serum at the recommended dosages of 0.5 cc. and 10.0 cc., with one 5-cc. dose of an adsorbed erysipelas bacterin, or with two doses of the bacterin at an interval of one month. Groups of the vaccinated pigs and of 63 unvaccinated controls were challenged at monthly intervals starting approximately two months following vaccination, through 6½ months of age. Challenge was performed by the skin scarification method, and criteria of immunity were cutaneous reactions using Shuman's classifications, temperature rise, and "stiffness." Results were examined statistically.

In all cases, more satisfactory results in protecting pigs to market age were obtained by the administration of one dose of bacterin than with culture and serum, but definite superiority was demonstrated by the administration of a second immunizing dose of bacterin one month following the first.

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Gaseous Requirements for Growth of *Vibrio Fetus*

C. V. REICH, M.S.; E. V. MORSE, D.V.M., Ph.D.; J. B. WILSON, Ph.D.

Madison, Wisconsin

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Present techniques for the cultivation of *Vibrio fetus* suggest the use of an atmosphere containing 10 per cent carbon dioxide.[1-3,5] The reason for the use of carbon dioxide is obscure and after a search of the literature we assumed that the procedure was developed as a result of the methods used for cultivation of *Brucella abortus*. Early investigators[4-6] discussed the similarity in the cultural and pathological features of *V. fetus* and *Br. abortus*. Later, when *Br. abortus* was shown to require carbon dioxide *per se*,[6-10] it is probable that the earlier comparison was sufficient to cause this method of cultivation of *Br. abortus* to be referred, unquestioned, to the cultivation of *V. fetus*.

This paper reports experiments designed to determine whether the use of 10 per cent carbon dioxide in the environment favors or stimulates the growth of *V. fetus*.

Materials and Methods

General Procedure.—The amount of growth on agar slants incubated in different gaseous environments was chosen as the criterion for determination of the most effective environment for *V. fetus*. Accordingly, a standard loopful (ca. 4 mm. diameter) of a 72-hour-old semisolid broth inoculum was used to streak the surface of an agar slant. After incubation at 37 C. for five days in the desired atmosphere, the growth was removed by washing the surface of the slants and was measured turbidimetrically.

Cultures.—Forty-two strains of Vibrio from a variety of sources were employed. The entire group was grown initially under 10 per cent carbon dioxide in air, at 37 C. on slants made from Albimi Brucella agar plus 0.5 per cent added agar to a final concentration of 2 per cent agar. After one week, 12 strains showed visible growth. These 12 plus one strain showing possible growth and two strains which did not show evidences of growth were selected. Purity of the culture was ascertained at frequent intervals by appearance of the characteristic growth pattern as well as by Gram staining and microscopic examination.

Inoculum.—Five milliliter of semisolid broth, prepared by adding 0.1 per cent agar to Albimi Brucella broth, were dispensed into test tubes (15 by 125 mm.) and sterilized in the autoclave at 15 lb. for twenty minutes. These were seeded twenty-four hours after preparation with 0.5 ml. of a well-shaken, two-week-old inoculum and incubated at 37 C. under 10 per cent carbon dioxide. At seventy-two hours, the tubes were shaken to assure uniform distribution of the organisms and used immediately to inoculate the slants. After this use, they were kept in air at room temperature, to be used for future seeding.

Agar Slants.—Fifteen milliliter of the modified Albimi Brucella agar medium were dispensed into test tubes (22 by 175 mm.). After sterilization in the autoclave, the tubes were slanted to give uniform surfaces about 10 cm. in length. The tubes were inoculated twenty-four hours after preparation by touching a standard loopful of inoculum to the surface 2 cm. from the leading edge, pushing forward to within 1 cm. of the bottom edge, and then withdrawing the loop by streaking from side to side to cover all of the surface. The lower 1 cm. of the slant surface was avoided in order to minimize the growth in the syneresis fluid.

Gaseous Environment.—Gas measurements were made at room temperature (ca. 25 C.) using a barometer and a mercury manometer. The inoculated agar slants were placed in vacuum desiccators; the latter were evacuated to the amount of substitute gas desired, plus the amount of barometric pressure over 700 mm. When the substitute gas was added, there remained a partial vacuum corresponding to the barometric pressure in excess of 700 mm. and the total pressure in the desiccators was constant.

Preliminary investigation showed that the carbon dioxide normally present in the air had no effect within the accuracy of the experiment and was ignored. All diluting gases were commercial preparations having an analysis of better than 99.5 per cent purity.

Turbidity Readings.—The inoculated agar slants were removed from the artificial environment after five days of incubation at 37 C. Three milliliter of 0.4 per cent formalin in sterile 0.85 per cent sodium chloride solution was added to each tube to wash the growth from the surface. These washings were placed in stoppered collection vials. Twenty-four hours later, 2-ml. aliquots were taken after uniform shaking and transferred to photometer tubes for turbidity readings. A Coleman junior spectrophotometer was used with light wave

From the Departments of Bacteriology and Veterinary Science, University of Wisconsin, Madison.

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Mr. Reich's present address is Department of Veterinary Science, Pennsylvania State University, State College.

Dr. Morse's present address is Department of Microbiology and Public Health, Michigan State University, East Lansing.

of 5,500 angstroms being selected as offering the broadest scale for reading. Readings are given as optical density x 100. When the results of the duplicates were in disagreement, a single culture of that strain was retested.

Catalase Test.—During the course of the investigation, the entire stock of cultures was examined for catalase activity. The 42 strains were inoculated onto agar slants and incubated as previously described, in an environment of 475 mm. of mercury of helium plus 225 mm. of mercury of air (6 % oxygen). Following incubation, a loopful of U.S.P. 3 per cent hydrogen peroxide was touched to the growth on each slant. If peroxide decomposition, as evidenced by bubbling, occurred, the strain was considered catalase positive.

Hydrogenase Test.—The total stock of 42 strains was grown on agar slants in an atmosphere of 350 mm. of mercury of hydrogen plus 350 mm. of mercury of air (9.2 % oxygen). After five days' incubation at 37 C., only those strains to be tested immediately were removed from the incubation environment. Suspensions of approximately 10^7 cells per milliliter in sterile 0.85 per cent sodium chloride solution were made of each strain to be tested.

The Klett-Summerson Thunberg tube method [9] was used for the test. Two ml. of M/15 potassium phosphate, adjusted to pH 8.0 with orthophosphoric acid, 0.7 ml. of a 1:10,000 aqueous dilution of methylene blue chloride and 1.3 ml. of distilled water were placed in each tube. Into the cap was placed 0.1 ml. of the cell suspension. The tubes were closed and evacuated with a water aspirator, then refilled with hydrogen. This procedure was repeated three times and the tubes were then placed in a 36 C. water bath and allowed to equilibrate for temperature, after which time the cells were tipped into the tube.

If decolorization of the methylene blue occurred, the procedure was repeated under the same conditions of the previous trial, but helium was used instead of hydrogen to replace the evacuated air. For the purpose of this work, positive hydrogenase activity is defined as the ability to reduce methylene blue within one hour under hydrogen gas and the failure to reduce the dye within two hours under helium.

RESULTS AND DISCUSSION

Data from the experiments on growth of *V. fetus* in different gaseous environments are given in table 1. The gaseous environment is given in percentage of oxygen in the diluting gas. It is apparent from these data, which are given in optical densities representing the comparative amounts of growth, that as the percentage of oxygen is lowered the growth increases to a point where a very low amount of oxygen becomes the limiting factor. Carbon dioxide as a diluting gas resulted in no better growth than when nitrogen, helium, or vacuum were used to dilute the oxygen which is proof that an increased percentage of carbon dioxide *per se* is not needed for growth but that carbon dioxide simply serves as a diluent to lower the percentage of oxygen in the environment. The commonly accepted environment of about 10 per cent carbon dioxide in the air is denoted in table 1 at 15.8 per cent of oxygen. It is evident that greater yields of cells can be obtained by using greater concentrations of diluent regardless of gas used. Moreover, carbon dioxide as diluent demonstrates some inhibition when in the higher concentrations. In general, all gases with the exception of hydrogen caused comparable growth curves.

TABLE 1—Mean, Maximum, and Minimum Growth of 15 Strains of Vibrio Fetus Under Different Gaseous Environments

	Oxygen remaining (%)	19.3	15.8	13.1	10.5	8.0	5.3	2.6	0.6
Diluent gas									
Air (partial vacuum)	maximum	38	54	65	75	83	(vacuum too great for container)		
	mean	8	19	29	66	71			
	minimum	1	1	1	5	55			
Hydrogen	maximum		92	103	109	103	105	75	31
	mean		64	78	85	62	80	26	5
	minimum		7	14	42	8	15	6	1
Nitrogen	maximum		61	49	74	79	81	62	39
	mean		26	42	56	66	68	45	12
	minimum		2	7	14	46	49	6	1
Helium	maximum		59	72	69	80	75	62	22
	mean		37	41	47	66	63	42	6
	minimum		5	12	7	44	38	7	1
Carbon dioxide	maximum		69	66	75	71	76	61	20
	mean		38	40	50	55	48	36	6
	minimum		2	1	7	14	6	1	1

Numbers represent optical density times 100.

The data show greatly increased maximum cell yield obtained under hydrogen as the diluting gas. This increase was observed in several strains only, while the remaining strains behaved as they did under the other gases.

Table 1 demonstrates the critical nature of the concentration of air in the incubation environment. The maximum cell yield evidently occurs within the range of 8 per cent oxygen. Using the values obtained in the experiments with helium, the maximum yield can be extrapolated (from a prepared curve) as occurring at 225 mm. mercury of air and 475 mm. mercury of diluent. If oxygen of the air is assumed to be the critical factor, this point then corresponds to an optimum oxygen level of 6 per cent. The values for hydrogen, carbon dioxide, and evacuation only were ignored in this determination in order to eliminate the possibilities of, respectively, stimulation, inhibition, or pressure effect. The oxygen level of 6 per cent was tested on all strains available (a total of 42) and these responded well to this environment, showing good cell yields on agar surfaces, in broth tubes, or in broth shake cultures.

The maximum yields reported (table 1) reflect the ability of a few strains to grow better in the medium particularly at the higher percentages of oxygen. This is probably in part an expression of the familiar observation that *V. fetus* may be "adapted" to growth in air, *i.e.*, a culture produces mutants which are better adapted to grow in air than the parent culture which grows best in lowered amounts of oxygen.

During the evaluation of the data, it became apparent that if the responses of individual strains were considered, then the 15 strains used could be divided into two groups. Each strain within a group would generally conform to a curve typical of that group, and still further, each group would generally include strains isolated from a common host species, *i.e.*, ovine or bovine. This manifestation becomes most apparent at the lowest percentage of oxygen used in the experiment. If the desiccator containing the inoculated slants is evacuated to 24 mm. of mercury of air remaining (0.6 % oxygen) and then nitrogen is added to a total pressure of 700 mm. of mercury, the division of the species becomes visually evident. The cell yield of the ovine strains is sufficient to cause visible turbidity while the bovine strains produce no turbidity. Thus, it appears that this may be a technique useful in identifying strains of *V. fetus* of bovine and ovine origin.

On examination of the results of these experiments, two supplementary questions arose. First, does the increased cell yield of certain strains in the presence of hydrogen indicate the presence of a hydrogenase? Second, could the growth of some strains at the near anaerobic conditions of the separatory technique be related to the absence of a catalase system?

The entire stock of 42 strains was tested for hydrogenase and catalase activity. Twenty-four of the 42 strains were positive for hydrogenase and 32 were positive for catalase. There was no apparent correlation between the occurrence of these enzymes. The presence of hydrogenase had no obvious relation to the origin of the culture but could be predicted with reasonable certainty on the basis of increased cell yield under hydrogen plus air when compared to cell yield at a comparable dilution of the air with any of the other gases. The test for catalase did not offer a means of separating ovine from bovine strains. It did, however, show specificity as to source of the isolate. Without exception, those ten strains which were isolated from vaginal mucus or bull semen did not possess catalase while the 32 strains directly connected with a clinical infection were catalase positive. The ten "saprophytic" strains along with 27 isolates from known bovine or ovine abortions were utilized for confirmation of the separation technique. Within the latter group, seven strains, of which six were ovine, showed visible turbidity and 20, of which 18 were bovine, showed no growth. This constitutes separation on the basis of known origin, using the low percentage of oxygen technique, of 24 of 27 strains with an accuracy of 90 per cent. The ten "saprophytic" strains from mucus or semen demonstrated no significant correlation. Moreover, even though all ten saprophytic strains were of bovine origin, every degree of growth from absence to luxuriant was evident within the group.

Since the completion of these investigations,[7] the reports of Bryner and Frank [2]

and Price *et al.*[6] have been published which also report similar findings regarding the catalase activity of *V. fetus* and related "saprophytic" vibrios.

SUMMARY

Carbon dioxide in concentration greater than that usually present in air is not essential to the growth of *Vibrio fetus*. Forty-two strains tested responded to a microaerophilic environment containing 6 per cent oxygen with considerably better growth than in air environment containing 10 per cent carbon dioxide. The percentage of oxygen is lowered using, as diluent, helium or nitrogen rather than carbon dioxide which exhibits some inhibition at this concentration. Many strains are apparently sensitive to hydrogen, showing increased yields in its presence. The ability to reduce buffered methylene blue in the presence of hydrogen but not helium was demonstrated by 24 of the 42 strains.

It was possible to divide the test group of organisms into three categories. Vibrios isolated from bovine semen or vaginal mucus were catalase negative as compared to the remainder of the group which were catalase positive. Ovine and bovine abortion isolates could be divided, according to origin, on the basis of amount of growth in an atmosphere of 24 mm. of mercury of air and 676 mm. of mercury of nitrogen. With a standardized inoculum, the ovine strains produced visible growth, while the bovine strains did not give visible growth.

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Jaundice and Bilirubinemia as Manifestations of Canine Distemper in Raccoons and Ferrets

LAWRENCE KILHAM, M.D.; ROBERT T. HABERMANN, D.V.M.; CARLTON M. HERMAN, D.Sc.

Bethesda, Maryland, and Laurel, Maryland

A PREVIOUS communication [1] described an agent, isolated from the liver of a wild raccoon *(Procyon lotor)*, which caused bilirubinemia and jaundice in raccoons and ferrets. Contrary to this preceding report, examination of more extensive material has revealed presence of inclusion bodies in various tissues, which are characteristic of canine distemper. Cross-immunity tests and clinical observations have further identified this icterogenic agent as a member of the canine distemper virus (DV) complex. Bilirubinemia has not been considered a usual manifestation of diseases induced by these viruses.[2] The present report describes isolation of two further strains of DV which have induced jaundice, one from a wild raccoon and one from ferrets, and describes some of their attributes.

METHODS

Raccoons and Ferrets.—Aside from a few purchased in Florida, raccoons were obtained from wildlife refuges in Louisiana, South Carolina, Delaware, and Maryland. Ferrets were purchased from dealers.

Safeguards Against Accidental Infections.—Canine distemper is a difficult infection to control and the following safeguards were observed. Noninfected and unused animals were kept on an animal farm ten miles from the laboratory building where inoculated animals were maintained in separate cages. Clean, cardboard containers were used for each food ration and animals were fed and watered as a separate operation after cage cleaning. Obviously ill animals were placed together in a separate room reserved for them.

Serums.—Blood was obtained by cardiac puncture. Serums were used fresh for bilirubin determination, inactivated at 56 C. prior to use in serological tests, and stored in a frozen state. In a number of instances, animals which had died of the disease were bled satisfactorily postmortem.

Virus Preparations.—Virus was stored by placing whole sections of infected livers and spleens in screw-top vials for preservation by freezing. For passage, aliquots of this material were thawed, made into 1:10 suspensions with meat infusion broth and inoculated in amounts of 1 ml., intraperitoneally. To remove bacteria, such preparations were centrifuged at 4,000 r.p.m. for one hour in a cold centrifuge and then either treated with penicillin and streptomycin or passed through a Selas .03 bacteria-tight filter. Two strains of DV, designed as R_1 (Maryland) and R_2 (Pennsylvania), have been isolated from wild raccoons. The R_1 strain was the subject of a previous report.[1] A third strain (F_1) was isolated from ferrets apparently shipped from Ohio when diseased.

Standard Virus and Control Serums.—Two strains of DV, which were of canine origin and had been passed in ferrets, were obtained from other laboratories. Strain 788 [3] was obtained from the Army Medical Service Graduate School, Washington, D. C. An additional strain was obtained from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., which also supplied paired ferret serums which included a known positive and a normal control as determined by neutralization tests run in ferrets against DV. Canine distemper vaccine, avianized (Lederle) given in doses of 1 to 2 ml. subcutaneously, was used in vaccination studies.

EXPERIMENTS AND RESULTS

Isolation of Virus from Wild Raccoons.—Distemper virus (R_2) was isolated from a diseased wild raccoon which was captured in Pennsylvania in May, 1954. A 10 per cent suspension of pooled liver and spleen from this animal was inoculated intraperitoneally in amounts of 1 to 2 ml. into 4 other raccoons. Two of these remained well and 2 became ill, both of the latter developing bilirubinemia. One of the latter raccoons became ill in seven days and was moribund with marked jaundice (total serum bilirubin 7.6) on the following day. The original raccoon (R_2) did not have bilirubinemia, but cytoplasmic inclusions were found in biliary epithelium similar to those photographed in the other (R_1) raccoon (fig. 1). Raccoon R_2 also had cytoplasmic inclusions in bronchial epithelium and intranuclear inclusions in glial cells of the cerebrum and in the granular layer of the cerebellum.

From the National Microbiological Institute (Kilham); National Institute of Arthritis and Metabolic Diseases (Habermann), National Institutes of Health, Bethesda, Md.; and Department of Interior, Fish and Wildlife Service, Patuxent Research Refuge (Herman), Laurel, Md.

Dr. Kilham is now at the Rocky Mountain Laboratory, Hamilton, Mont.

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Type of Disease Induced in Raccoons and Ferrets.—In present experiments, the R_1, R_2, and F_1 strains of virus have given a similar clinical picture in raccoons. Continued observations on over 50 animals have added little to the original description,[1] although hyperemia and edema of anal, gingival, and buccal mucosae as well as characteristic odor have been pronounced in some cases. Convulsive movements, observed in the raccoon from which the R_1 strain was isolated, have not been encountered in animals artificially inoculated. Ferrets inoculated in present experiments with the R_1 strain usually died within 1 to 3 days, presenting minimal conjunctivitis and mucosal lesions. Findings more commonly associated with distemper in ferrets were not observed until the apparent introduction, late in the course of present experiments, of the F_1 strain of DV.

At this time, a shipment of 30 ferrets was received. Of these, 17, mostly pregnant females, developed paralysis and encephalitis whether or not they had been inoculated. Of particular interest was the observation that in continued ferret-to-ferret passage by means of infected liver suspensions, many of those inoculated developed stomatitis, swelling and closure of the eyelids with exudate and, as discussed below, higher levels of serum bilirubin than had been encountered previously. Two raccoons which became ill 11 days after inoculation with the F_1 strain developed marked jaundice. In the infections induced by all 3 strains (R_1, R_2, F_1), enlargement of spleens has been striking in ferrets but not in raccoons. Catarrhal inflammation and pneumonitis have been minimal in both species. Approximately 100 per cent of ferrets and 50 per cent of raccoons have proved susceptible in present experiments to fatal disease following inoculation with any one of the three strains. The minimum incubation period for all strains was eight to nine days (table 1).

Bilirubinemia.—Levels of serum bilirubin in 39 raccoons and 23 ferrets which were bled when moribund or recently dead from distemper are summarized (table 1).* Serums obtained from these raccoons and ferrets prior to inoculation with virus had total bilirubins ranging from 0.01 to 0.29 mg./100 ml. A level of 0.3 mg./100 ml., therefore, has been regarded as the upper limit of normal. A majority of diseased raccoons inoculated with the R_1 strain (experiment 1) had total serum bilirubins of from one to four at time of death, 14 of 19 in this group having had jaundice. The highest bilirubin encountered was 11.8 mg./100 ml. Of 3 raccoons infected with the R_2 strain (experiment 2), 2 developed jaundice. The 12 ferrets inoculated with the R_1 strain of DV (experiment 3) developed mild bilirubinemia, 9 having had levels below 1.0 mg./100 ml. and 3 below 2.2 mg./100 ml. when moribund or recently dead from infection. The majority of 11 ferrets inoculated with the F_1 strain (experiment 4) had a pronounced bilirubinemia. Five had a total serum bilirubin higher than eight, and 2 had levels of 15.1 and 18.4.

Cross-Immunity Tests.—Both adult raccoons and weanling ferrets were vaccinated by subcutaneous inoculation of 1 to 2 ml. of avianized distemper virus (Le-

Fig. 1—Section of liver from wild raccoon (R_1) showing cytoplasmic inclusions of bile duct epithelium (arrow). Hematoxylin and eosin. x 750.

* Determinations performed in laboratory of Dr. Roderick Murray, Biologics Control Laboratory, National Microbiological Institute, National Institutes of Health, Bethesda, Md.

TABLE 1—Distribution of Elevated Levels of Serum Bilirubin among Raccoons and Ferrets Bled when Moribund or Recently Dead from Distemper

Experiment	Species	Strain of DV* used for inoculation†	Number of Animals							Time (days)	
			Inoculated	Total serum bilirubins (mg./100 ml.)					With jaundice	Incubation period	Duration of illness
				0.3-1**	1-4	4-8	8-12	12-18			
1	Raccoon	R_1	36	10	19	5	2	None	21	8–20	2–8
2	Raccoon	R_2	3	1	None	2	None	None	2	8–16	2–10
3	Ferret	R_1	12	9	3	None	None	None	None	9–13	1–3
4	Ferret	F_1	11	4	2	None	3	2	1	8–15	1–5

* Distemper virus; † all animals inoculated with 1:10 suspension of infected livers and spleens, 1 to 2 ml. intraperitoneally; ** 0.3 mg./100 ml. considered upper limit of normal.

derle). Vaccination under these conditions led to immunity in most but not all of the ferrets. Thus, 3 of 6 ferrets challenged with one strain (788) of DV of canine origin two weeks after vaccination, developed signs characteristic of distemper and were dead or moribund nine to 13 days later. Likewise, 3 of 10 ferrets challenged with a second strain of DV (Lederle) of canine origin, became ill. One of these was moribund in nine days and the other 2, which were mildly ill in ten to 12 days, were killed when bled six to eight days later. There remained, therefore, 10 vaccinated ferrets which had resisted challenge with strains of DV obtained from other laboratories (group 1, table 2). These animals, two weeks after their initial challenge, were given a final challenge with the F_1, R_1, and R_2 strains. Animals which survived without illness over a three and one half-week to five-week period of observation were considered to have resisted challenge. Of 3 ferrets which resisted challenge with DV strain 788, one resisted subsequent challenge with the R_1 and 2 with the F_1 strain. Of 7 ferrets unaffected when challenged with the Lederle strain, 3 survived subsequent inoculation with the R_1 strain, 2 with the R_2 strain, but only 1 of 2 given a final challenge with the F_1 strain remained well. The ferret which became ill 12 days after inoculation with the F_1 strain and died when bled two days later had swelling and closure of the eyelids.

Additional cross-immunity tests were performed in animals vaccinated but not subsequently challenged with DV of canine origin. Of 5 raccoons (group 2, table 2) inoculated three weeks after vaccination, 3 remained well following challenge with the F_1 and 2 following challenge with the R_2 strain. Seven ferrets (group 3, table 2) were challenged 3 weeks after vaccination, 3 with the R_1 and 4 with the F_1 strain. Six survived this challenge without illness, and 1 became ill 15 days after in-

TABLE 2—Results of Challenge of Distemper Vaccinated Ferrets and Raccoons with Bilirubinemia-Inducing Strains of Distemper Virus (DV)

Group	Species and No. involved	Date vaccinated (avian vaccine)	Challenge with DV strain canine origin		Final challenge with bilirubinemia-inducing strains			Results of final challenge	
			Strain	Date	Strain	No. of animals	Date	Remained well	Died or were killed when moribund
1	3 ferrets	5/11	788	5/24	R_1	1	6/10	1	0
					F_1	2		2	0
	7 ferrets	5/25	Lederle	6/9	R_1	3	6/23	3	0
					R_2	2		2	0
					F_1	2		1	1
2	5 raccoons	5/13	.....	ND*	F_1	3	6/3	3	0
					R_2	2		2	0
3	7 ferrets	5/11	.....	ND	R_1	3	6/1	3	0
					F_1	4		3	1 ill (atypical)
4 (controls)	4 ferrets	ND*	.....	ND	R_1	2	6/3	0	2
					R_2	1		0	1
					F_1	1		0	1
5 (controls)	3 raccoons	ND	.....	ND	R_1	1	6/3	1	0
					R_2	2		0	2

* Not done.

oculation with the F_1 strain. This sick ferret circled or rolled over and over in one direction, a form of disease not observed in other ferrets. The R_1, R_2, and F_1 virus preparations used in cross-immunity tests were fully virulent as demonstrated by the disease and deaths induced in unvaccinated control animals, which included 4 ferrets and 3 raccoons (groups 4 and 5, table 2).

DISCUSSION

The term "canine distemper virus complex" would appear a useful one to cover strain differences manifested by variations of disease patterns induced by closely related agents. In this respect, these agents may be compared to the psittacosis group of viruses. Inclusion bodies, cross-immunity, and possibly complement-fixation tests at present under study may provide common denominators for the entire complex. The above studies have centered on three freshly isolated strains of DV all capable of inducing bilirubinemia. Three lines of evidence, clinical picture, pathological changes, and cross-immunity tests, have indicated that the bilirubinemia-inducing agents are members of the canine distemper complex. Diseases induced in ferrets by the R_1, R_2, and F_1 strains correspond to descriptions given by Larin [5] and his so-called group A strains of DV. Differences in incubation period and duration of illness may have resulted from the fact that febrile reactions were not determined in present investigations. Description of distemper in ferrets by Dunkin and Laidlaw [6] is similar in some respects to illnesses described above especially in regard to incubation period and appearance of mouth and eyes. Swelling of the feet, however, was observed in only a small number of ferrets, all infected with the F_1 strain. Thus far, we have found no full descriptions of distemper in raccoons.

Location, distribution, and appearance of inclusion bodies encountered in experimental animals and in 2 original raccoons from which the R_1 and R_2 strains were isolated did not differ from descriptions by Green and Evans.[4] Presence of cytoplasmic inclusions, seen on histological sections of biliary epithelium, suggest that this tissue is subject to infection with DV. Hepatic cord cells have contained no inclusions and except for scattered areas of focal necrosis, have appeared relatively undisturbed. Jaundice has not been as obvious in ferrets as in raccoons, yet the former have exhibited higher levels of serum bilirubin. An explanation of this phenomenon may be that raccoon fat is normally white so that a yellow tinge is readily observed. Ferret fat, on the other hand, is normally rather pink and offers less suitable background.

In reviewing present investigations, it would appear that host factors may influence the type of disease induced in ferrets by DV. Thus, the F_1 strain was originally recovered from a shipment of ferrets, most of which were pregnant. The fact that 17 of 30 of these ferrets developed paralysis of hind quarters and convulsions, whereas passage of virus to other ferrets, whether males or nonpregnant females, failed to produce this type of illness suggests that pregnancy may be a factor predisposing to DV-encephalitis. A second host factor not properly evaluated in present studies is that of age. Most of the ferrets observed for bilirubinemia induced by the R_1 and F_1 strains appeared to be adults. On the other hand, ferrets available for inoculation with strains of canine origin were only one or two months beyond the weanling stage as was a single animal inoculated with the R_2 strain. Until the question of whether young ferrets are less susceptible than adults to bilirubinemia is answered, it would appear preferable to use older animals for investigations of this manifestation of distemper.

Over-all results of cross-immunity tests provide evidence that the bilirubinemia-inducing strains are part of the canine distemper complex. Thus, of 10 ferrets which had been previously vaccinated and tested for immunity by subsequent challenge with DV strains of canine origin, 9 resisted challenge with the R_1, R_2, and F_1 strains. Of 5 raccoons and 7 ferrets vaccinated three weeks previously, all but 1 ferret resisted challenge with the bilirubinemia-inducing strains. R_1, R_2, and F_1 virus preparations as well as those of the canine strains used for challenge in these tests were shown to be fully virulent by deaths induced in control animals. An unanticipated development in these experiments was that the distemper vaccine did not protect all ferrets, particularly those

challenged with strains of canine origin. More complete protection might have been obtained if the ferrets used, which were small weanlings, had been older.

In conclusion, an idea that distemper may be of importance as a cause of epizootics among wild carnivores, such as are known to reduce raccoon and skunk populations in certain localities, would appear to offer, on the basis of present findings, a basis for future field investigations. Diagnosis of DV infections in wild life would be most certain when clinical and pathological observations, coupled with isolation of virus, all provided confirmatory evidence.

SUMMARY

1) Two strains of distemper virus have been isolated from wild raccoons and one strain from ferrets.

2) All strains isolated have induced bilirubinemia in raccoons and ferrets. Many raccoons with bilirubinemia also had jaundice.

3) Identification of these strains as members of the canine distemper virus complex has been by clinical and pathological findings consistent with this diagnosis as well as by cross-immunity tests.

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ADDENDUM

Since preparation of the manuscript, the complement-fixation tests referred to in the discussion have been shown to be specific and results will be published. They give added proof that the R_1, R_2, and F_1 strains are part of the distemper virus complex.

Studies on Depression of Radioiodine Uptake by the Thyroid After Phenothiazine Administration. I. Investigations of the Factors in Phenothiazine N. F. Responsible for the Thyroidal Effects

R. H. WASSERMAN, Ph.D.; B. F. TRUM, D.V.M.; R. A. MONROE, Ph.D.; J. J. LANE, B.S.; C. L. COMAR, Ph.D.

Oak Ridge, Tennessee

PHENOTHIAZINE (thiodiphenylamine) N. F. (green),* one of the most useful veterinary anthelminthics, has been shown by Talmage *et al.*[1-3] to suppress I-131 (radioiodine) uptake by the thyroid gland of the rat, burro, sheep, ox, and other species. Because it has been demonstrated that minute quantities of iodine are able to decrease thyroidal uptake of I-131,[4,5] Talmage and his group *(loc. cit.)* have suggested that the iodine known to be present in green forms of phenothiazine may produce the observed results by iodine saturation of the thyroid gland or dilution of the radioisotope. The possibility of a true thyroid depressor in phenothiazine was not excluded. The purpose of this work was to investigate the validity of either postulate.

Radioiodine is employed clinically to assess the ability of the thyroid to accumulate iodine, to incorporate iodine into thyroxine, and to release thyroid hormone into the circulatory system.[6] A lowered thyroidal uptake of I-131 may be indicative of an inhibition of the accumulating mechanism in the thyroid *per se*, a suppression of pituitary gland (thyrotropin production) or the action of an increase of stable iodine in the blood.

The chemical, chromatographic, and physiological behavior of iodine in commercially available phenothiazine and laboratory-synthesized phenothiazine was compared with potassium iodide. In addition, the thyroidal effects of a number of commercial phenothiazines, both purified and green, were assayed. These studies indicate that at least two factors are operative in depressing thyroidal I-131 accumulation.

This study was performed at the University of Tennessee-Atomic Energy Commission Agricultural Research Program, Oak Ridge, and was supported by a grant from the Grayson Research Foundation. Approved for publication by the director of the University of Tennessee Agricultural Experiment Station.

Drs. Wasserman and Comar are now at the Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn. Dr. Trum is Lieutenant Colonel, Veterinary Corps, United States Army. Mr. Lane is a research associate in the Program. Dr. Monroe is now at the University of California, Atomic Energy Project, Los Angeles 24.

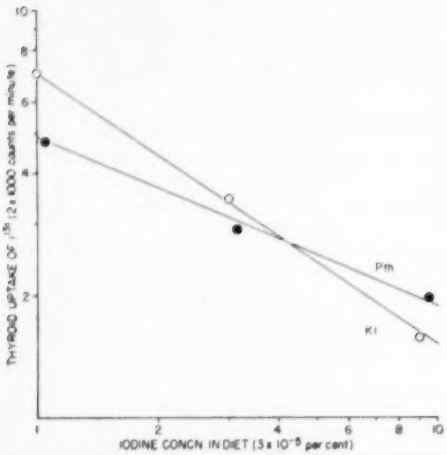
* Phenothiazine N.F. (green) is the nonpurified commercial product with a purity of about 98 per cent and freezing point of not less than 179 C. Purification by crystallization, sublimation, et cetera, yields the pale yellow purified phenothiazine with a melting point about 184-185 C., labelled phenothiazine N.F. (purified).

METHODS AND EXPERIMENTAL DATA

Comparison of Potassium Iodide (KI) and Phenothiazine N. F. (Green) on Thyroidal I-131 Uptake.—Phenothiazine N. F. (green) and KI, at equivalent iodine levels (3×10^{-8}, 9×10^{-8}, and 30×10^{-8} %), were compared. The thyroidal I-131 accumulation should have been similar if the iodine content of phenothiazine was the sole causative agent. The bioassay of the thyroid effects of these substances involved the utilization of groups of 10-day-old chicks maintained on a low iodine, corn meal ration for three days, followed by three days on their respective experimental rations. The chicks were then injected intraperitoneally with 0.2 μc. of carrier-free I-131 as the iodide ion. Eight hours after the injection of I-131, the chicks were killed and their thyroid glands excised for radioassay. Each group contained 18 chicks.

Statistical analysis of the results (graph 1) showed the thyroid uptake vs. iodine concentration curves for both treatments to be straight line functions on a log-log plot. The two curves were in proximity, crossing at an iodine concentration of approximately 12×10^{-5} per cent. However, the slopes of the lines differed by a small but statistically significant value ($p < .01$). These observations suggest that the major responsible agent in commercial phenothiazine could be iodine but that still another factor contained therein slightly altered thyroidal I-131 uptake.

Comparative Effect of Phenothiazine from Different Sources on Thyroidal I-131 Uptake.—Five phenothiazines N. F. (green) and two purified phenothiazines N. F. were compared as to their ability to depress thyroidal I-131 uptake by the aforementioned method. The purpose of this survey was to test the uniformity of the thyroidal response to different phenothiazines. If iodine alone is the causative agent, variations in the ef-



Graph 1—Comparative effect of potassium iodide (KI) and phenothiazine (PTH) N.F. (green) on thyroidal radioiodine (I-131) accumulation.

fects of these drugs on the thyroid should be reflected by a similar variation in iodine content.

The phenothiazines were purchased or donated by various companies from open stock unless otherwise specified.

The details and results of this experiment are shown (table 1). All of the green phenothiazines significantly decreased thyroidal I-131 uptake. It is noteworthy that one preparation (PTH B) showed a much greater depression of thyroidal uptake than would be expected from their comparative dietary iodine values. This suggested that factors other than iodine intake might be operative, at least in the sample which elicited the divergent response.

The purified phenothiazines N. F. (F, G), at a dietary level of 0.1 per cent, reduced thyroidal I 131 uptake to a slight but significant extent (table 1). Both of these purified samples were relatively free of iodine, containing less than 10^{-4} per cent. Collier and Allenby[7] have reported that phenothiazine and certain phenothiazine derivatives interfere with various oxidative enzyme systems. It was not determined whether the thyroid effect of purified phenothiazine was due to a general enzyme inhibition or a specific effect on the thyroid. The former possibility offers an attractive explanation for its action. Again these data suggest a mechanism independent of iodine intake.

TABLE 1—The Effect of Phenothiazine on the Uptake of Radioiodine by the Thyroid Gland

Supplement	Dietary level (%)	No. of chicks	Total dietary iodine (μg./100 Gm.)	Thyroidal I-131 uptake (% of control)
Low iodine controls	.....	19	4	100
PTH A (green)**	0.05	18	174	28*
PTH B (green)	0.05	18	199	10*†
PTH C (green)	0.05	18	179	27*
PTH D (green)	0.05	19	164	21*
PTH E (green)	0.05	10	174	24*
PTH F (purified)‡	0.10	18	4	76*
PTH G (purified)	0.10	18	4	71*

* Significantly different from control at $p < .01$; ** PTH (green)—phenothiazine N.F. (green); † significantly different from PTH's A, C, D, and E at $p < .01$; ‡ PTH (purified)—purified phenothiazine N.F.

Synthesis of Phenothiazine with Labelled I, Catalyst, and the Thyroid Uptake of the Labelled Iodine Product.—The laboratory synthesis of phenothiazine in the presence of radioiodine catalyst provided a method whereby the comparative thyroid uptake of the iodine in the phenothiazine preparation and potassium iodide could be measured. The proportion of the reactants and the conditions of the reaction in the synthesis of phenothiazine were those found by Belokrenitshii[8] to yield optimal quantities of product. The reaction was assumed to have gone to completion when H_2S evolution ceased.

The I_2* catalyst was prepared by adding KI* containing 6.5 μc. of I-131 (as KI) per 4.3 mg. of carrier KI to the stoichiometric amount of KIO_3 in dilute HCl and removed from the aqueous solution by ethyl ether extraction. The ether extract was quantitatively added to a homogeneous mixture of 3.00 Gm. of diphenylamine and 1.14 Gm. of sulfur. The reaction progressed rapidly as the temperature was increased gradually from 160 to 200 C. The melting range of the crude product was 170 to 178 C., while that of the commercial phenothiazine was found to be 175 to 183 C. No attempt at purification was made since purification processes may have removed or changed the state of the resultant iodine. The crude phenothiazine was suspended in water with the aid of tween 80.

Equivalent iodine levels of the original KI* solution and the labelled phenothiazine suspension were administered to comparable groups of 4 adult female albino rats maintained on a low iodine diet.* The rats were killed 20 hours post-administration and the thyroids removed for radioactive assay.

The thyroid glands of the KI*-administered group accumulated 6.9 ± 1.3† per cent of the total dose, while the phenothiazine-treated group accumulated 6.4 ± 1.3† per cent. Thus, no difference was observed in the ability of the thyroids to

† Mean ± standard error of the mean.
* Radioactive.

accumulate either iodide I-131 or the I-131 in the phenothiazine preparation. The quantitative similarity of these results is strong evidence that isotope dilution or thyroid gland saturation by the iodine in phenothiazine N. F. (green) could account for the suppression of thyroidal I-131 uptake.

Solubility and Chromatographic Properties of the Iodine in Commercial Phenothiazine N. F. (green).—The previous findings suggested that readily available inorganic iodine in green phenothiazine was responsible for the observed thyroidal effects. The validity of this hypothesis was further tested by examining certain properties of the phenothiazine iodine. These included a comparison of the solubility and chromatographic characteristics of potassium iodide with that of iodine in phenothiazine.

Twenty gram of phenothiazine N. F. (green), dissolved in a mixture of 200 ml. of *n*-butanol and 300 ml. of toluene, was extracted six times with 75 ml. of distilled water each time. The total iodine in aliquots of the original phenothiazine solution, the water-extracted phenothiazine solution, and the aqueous extract was determined. Equivalent volumes of three fractions were injected subcutaneously into chicks maintained on a low level iodine diet. Simultaneously, control chicks were injected with the solvent alone or with a KI solution containing iodine equal to the iodine level in the original phenothiazine solution. The thyroid uptake of I-131 was determined by the previously described method.

It was observed that almost all of the iodine in commercial green phenothiazine was extractable by water (table 2), suggesting the iodine to be inorganic in nature. The depression of thyroidal I-131 uptake was correlated with the iodine content of the three fractions. Nachimson *et al.*[10] have reported that, of four phenothiazine fractions collected from an activated alumina chromatographic column, the fraction containing the most iodine was the only one inhibiting I-131 uptake by the thyroid. Wasserman[11] separated phenothiazine into twenty fractions from an alumina column and found that only those fractions containing significant amounts of iodine affected the thyroid uptake of I-131.

By descending chromatographic procedures, the iodine in the aqueous extract of commercial phenothiazine N. F. (green) was compared with KI. The developing solvent systems were of the following composition: solvent A = butanol-1.5 N NH_4OH (1:1 v/v); solvent B = pyridine-butanol-1.5 N NH_4OH (2:1:1 v/v); solvent C = ethanol-conc. NH_4OH (98:2 v/v). In the case of solvent A, the components were immiscible and the organic layer was used. The location of the spots was determined by spraying the dried, developed chromatograms with a 1 per cent starch solution containing 2 per cent H_2O_2.

The paper chromatographic behavior of the iodine in KI and phenothiazine N. F. (green), as shown by their Rf values, was nearly identical with the three solvents employed (table 2). Although chromatographic evidence is not positive proof of the identity of unknown substances, these observations are highly indicative of a close chemical or physical relationship, or both, between KI and phenothiazine iodine.

Discussion

In these studies, the principal causative agent responsible for the observed thyroidal effect of phenothiazine seems to be the iodide ion. The evidences for this postulate were the similarity between the thyroid uptake of iodide I-131 and the radioiodine in the laboratory-synthesized phenothiazine preparation, and the chemical and chromatographic properties of the iodine in phenothiazine N. F. (green). Theoretically a consideration of the reaction by which phenothiazine is commonly synthesized also supports this finding since the initial production of hydrogen sulfide would immediately reduce any residual iodine (I_2) catalyst to the iodide ion. The formation of a phenothiazonium salt with I_2 would also yield the iodide ion.

There was a greater inhibition of thyroidal I-131 uptake by one of the five phenothiazines N. F. (green) tested, a small but significant slope difference resulting from the green phenothiazine-KI comparison and a slight depression by purified phenothiazine *per se*. This evidence pointed to at least another depressant of

TABLE 2.—Fractionation and Chromatographic Characteristics of Iodine in Phenothiazine N. F. (Green), and Effect of Fractions and Potassium Iodide (KI) on Thyroidal I-131 Uptake

Test substance	Total iodine in fraction (μg./ml.)	Uptake of I-131 by total thyroid gland (counts/min.)	Rf values of KI and fraction B solvent*		
			A	B	C
Control	—	1863(4)**	—	—	—
Total PTH†	140	131(4)	—	—	—
PTH Fraction A†	5	1715(3)	—	—	—
PTH Fraction B†	137	143(4)	0.20	0.61	0.51
KI	140	162(5)	0.20	0.63	0.46

* *See* text for description of solvents; ** No. of experimental animals in parenthesis.

† Total PTH—original solution of phenothiazine N.F. (green) prior to fractionation; PTH Fraction A—residue following aqueous extraction; PTH Fraction B—aqueous extract of phenothiazine solution.

thyroidal I-131 uptake in phenothiazine N. F. (green). However, these effects were small as compared to the aforementioned iodide effect.

Nachimson *et al.*,[10] following a series of experiments on the effect of phenothiazine on the thyroids in rats, have concluded that the thyroid depressant action is not due solely to the availability of free iodide, and suggested that an unknown organic compound or group of compounds in commercial phenothiazine is capable of suppressing the uptake of radioiodine by the thyroid. The present study indicated that the major depressant of thyroidal I-131 uptake in phenothiazine N. F. (green) is the iodide ion, and that other factors, much less potent than iodide, are also operative in reducing the accumulation of I-131 by the thyroid gland.

SUMMARY

1) The causative agent in phenothiazine N. F. (green) that suppresses the accumulation of radioiodine by the thyroid has been investigated.

2) In comparing the action of (potassium iodide) KI with phenothiazine N. F. (green), it was observed that their effects were grossly similar. However, the slopes of the thyroid uptake vs. dietary iodine concentration curves were different by a slight but statistically significant value.

3) One of five commercial phenothiazines from different companies caused a significantly greater thyroid depression than the others. Purified phenothiazine N. F. produced a small but definite inhibition of thyroidal I-131 uptake.

4) Phenothiazine was synthesized with labelled I_2 catalyst. The thyroid uptake of the labelled iodine in phenothiazine preparation was equal to that of labelled KI.

5) The iodine in commercial phenothiazine was almost completely soluble in water and chromatographically indistinguishable from KI.

6) The evidence suggested that the principal factor in phenothiazine responsible for the thyroidal response was the iodide ion, and the mechanism of action was by isotope dilution or thyroid saturation by iodide. Another factor, much less potent than iodine, was also responsible for a slight but definite depression of thyroid uptake of radioiodine.

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Studies on an Anthrax Vaccine Prepared from Nonencapsulated Variants of *Bacillus Anthracis*

G. PERSONEUS, B.S.; M. S. COOPER, Ph.D.; R. C. PERCIVAL, V.M.D.

Pearl River, New York

History

The etiological agent of anthrax was conclusively demonstrated by Robert Koch in 1877.[1] Perhaps a century prior to this time, however, a disease not unlike anthrax was described. This early history is reported in detail by Topley and Wilson.[2] Today, anthrax is a disease of primary importance throughout the world. In the United States, it is an ever-present danger to livestock, and vaccination is practiced in many areas.

In 1881, Pasteur[3] carried out the classic study at Pouilly-le-Fort. He succeeded in immunizing sheep with cultures that were altered in virulence by extended incubation at about 42 C. This method of vaccination was subsequently practiced on an extensive scale with millions of animals injected. Although the results obtained were in general satisfactory, the Pasteur vaccine had certain serious disadvantages. Apparently, exposure to elevated temperatures increased the frequency of appearance of avirulent mutants. In time these cells represented a good part of the suspension. Such a phenomenon, however, can not be easily controlled and, on occasion, vaccine of appreciable virulence was produced with some postvaccinal deaths as a result.

Sterne[4] has reviewed the introduction of the glucoside saponin in anthrax vaccine. Vaccines containing saponin have been prepared for many years. Cultures of stabilized and reduced virulence, as measured by their pathogenicity for mice, guinea pigs, and rabbits, have been used. A vaccine of this type was described by Eichhorn and Lyon.[5]

Anthrax vaccines prepared from cultures of reduced virulence and containing saponin are certainly an improvement over the original Pasteur method, but they are still not without disadvantages. Although difficulty is seldom experienced in the vaccination of cattle, some breeds of sheep and goats are extremely sensitive and postvaccinal losses may be quite severe. In our studies, we have seen sheep succumb to a vaccine prepared from a strain of organism not virulent for rabbits. The choice of a strain of *Bacillus anthracis* of still further reduced virulence, however, resulted in nonimmune animals as determined by challenge results. As a result of these difficulties, we became seriously interested in the type of vaccine prepared by Sterne.[6]

Some of the extensive studies necessary to develop this product[*] will be reported here. The problems encountered may perhaps serve to point out some of the peculiarities of immunity to this disease.

Production of the Vaccine

The procedures generally used were as described by Sutton.[7] A dried ampule of the Onderstepoort strain 34F2 was used to prepare a large number of dried stock cultures. Roux bottles were used for production flasks in which the area of the agar surface was about 200 sq. cm. The incubation period was 48 to 72 hours, and growth was harvested with glycerine-saline.

Testing of the Vaccine in Laboratory Animals

The demonstration of immunity in guinea pigs to challenge with *B. anthracis* is a difficult matter. Alexander[8] pointed out that, although such a test is successfully carried out in Onderstepoort, other laboratories have not obtained satisfactory results. Below is a resumé of our experience with this procedure.

Early in these studies, an occasional death occurred after vaccination when the stock suspensions were diluted with glycerin-saline. This was avoided by using only physiological saline.

Methods

Guinea pigs of the Hartley strain, raised in our own colony, and weighing between 400 and 500 Gm., were used exclusively in these studies. Vaccine was administered subcutaneously on the abdomen in a dose of 1 ml. The concentration of cells in the 1-ml. dose was 5×10^4, 5×10^6, or 10^7 (table 1).

The challenge dose, in all cases, was 1 ml. administered subcutaneously in the hindleg. Cultures used for challenge were the Pasteur No. 2-type and No. 3-type, prepared as follows:

Spores were grown on casein digest agar for 72 hours, harvested with 40 per cent glycerine-saline, and the suspension was heated at 65 C. for a half hour. Pasteur No. 2-type of culture suspensions contained 1.5×10^7 spores per milliliter, representing about 20 to 30 minimum lethal doses (m.l.d.) in 26 tests, and a preparation of the same concentration but of reduced virulence, representing 1 m.l.d., in 9 tests (table 1).

[*] Anthrax spore vaccine, carbozoo E nonencapsulated.

From the Biological Development Laboratory, American Cyanamid Company, Lederle Laboratories, Pearl River, N. Y.

The authors are indebted to Mrs. D. Budd for assistance in the preparation of the manuscript.

Preliminary studies with the Pasteur No. 3-type of culture in a liquid state resulted in higher survivals of vaccinated animals. Therefore, in order to develop a uniform standard challenge, Pasteur No. 3-type of culture was grown, harvested, and heat-treated as described. It was concentrated by centrifugation, resuspended to half the original volume with 10 per cent skim milk and frozen and dried in 10-ml. ampules containing 1 ml. of material. One per cent peptone water was used for reconstitution before use, and the resulting suspension contained 5×10^7 spores, representing approximately 10 m.l.d.

Results were recorded ten days postchallenge (table 1).

SUMMARY OF RESULTS

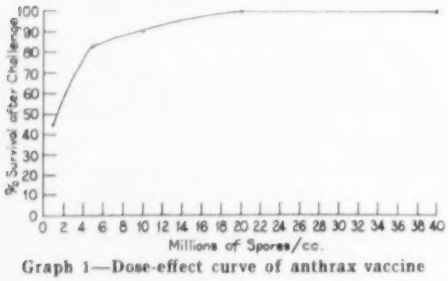
The data (table 1) were compared by *chi*-square with conclusions as follows:

1) The concentration of cells in the vaccine in the range studied had no effect on the survival rate in all groups (5×10^4, 5×10^6, 10^7).

2) The survival rate in group E was significantly higher than in all other groups.

3) The survival rate in group B was significantly higher than in groups A, C, or D.

4) The survival rate in group D was not different from group A or C.



Graph 1—Dose-effect curve of anthrax vaccine

Therefore, factors which influenced the survival after challenge included the number of vaccine doses given and the Pasteur type of challenge used. On the other hand survival after challenge was not affected by the concentration of cells in dose of vaccine between 5×10^4 and 10^7, nor by the number of lethal doses present in the Pasteur No. 2-type challenge within the range studied.

FURTHER IMMUNITY STUDIES IN GUINEA PIGS

The selection of a satisfactory challenge culture described in the previous section permitted further studies of the vaccine.

A number of tests to establish a dose-effect curve for the purposes of quality control yielded some very interesting results.

METHOD

Guinea pigs were vaccinated with 1 dose of vaccine as described. Twelve tests were performed on 260 guinea pigs to determine survival after vaccination with 1, 5, 10, 20, and 40 million viable spores per milliliter.

To insure a stable and uniform challenge preparation, a large number of vials of challenge culture were prepared from the Pasteur No. 3 strain. Each guinea pig was challenged 21 days postvaccination with 1 ml. of a reconstituted frozen and dried suspension containing 500,000 spores, representing about 10 m.l.d.

RESULTS

The average survivals at each dose concentration studied (graph 1) shows that almost complete protection resulted from injection with vaccine containing between 10 million and 40 million spores. The survival rate began to decrease perceptibly at 5 million spores, and at 1 million spores less than 50 per cent of vaccinated animals survived.

TABLE 1—Summary of Anthrax Immunity Studies in Guinea Pigs

Group	No. of tests	Challenge: No. of doses of vaccine	Challenge: Interval between doses	Challenge: Days after last immunizing dose	Challenge: Culture	Test Results[a] Survivors/total vaccinated, Vaccination dose (No. of spores): 5×10^4	5×10^6	10^7
A	15	1	.....	21	Pasteur No. 2[b]	21/77	25/81	31/74
B	5	2	7 days	14	Pasteur No. 2[b]	9/29	13/26	16/24
C	6	1	.....	21	Pasteur No. 2[b]	.....	26/118	.....
D	9	1	.....	21	Pasteur No. 2[c]	.....	39/152	37/105
E	21	1	.....	21	Pasteur No. 3[d]	.....	75/90	143/170

[a] In each test reported, at least 1 minimum lethal dose (m.l.d.) was used for challenge as established by 100 per cent deaths in a control group of 6 animals per test.

[b] 1.5×10^3 spores, representing 20–30 m.l.d. (1 m.l.d. = 500,000 spores).

[c] 1.5×10^3 spores, but milder than [b], representing approximately 1 m.l.d.

[d] 5×10^3 spores, representing about 10 m.l.d. (1 m.l.d. = 50,000 spores).

Safety and Immunity Studies in Sheep and Goats

The efficacy of this vaccine in domestic animals has been well established by Sterne[9] in South Africa.

We attempted studies in swine and calves but were unable to routinely produce a lethal infection in these two species; therefore, we have since restricted our investigations to sheep and goats.

Safety Tests

A number of animals have been vaccinated subcutaneously with 1 billion to 10 billion viable spores. The concentration usually averaged about 1 billion cells per milliliter. The recommended level for vaccination is 20 million spores. Therefore, these test animals have received 50 to 500 times the recommended dose.

A summary of safety tests performed during the routine production of vaccine is shown (table 2). A total of 34 sheep and goats received at least 1 billion viable cells each in the vaccine dose and all survived.

TABLE 2—Anthrax Vaccine Safety Tests in Sheep and Goats

Exptl. vaccine (lot No.)	No. of animals	Vaccination dose (billion spores)	Disposition
23	2 Sheep	10.0	Survived
27	1 Goat	10.0	Survived
42	1 Goat	10.0	Survived
43	1 Goat	10.0	Survived
45	1 Goat	10.0	Survived
Comb. 3/53	2 Goats	1.0	Survived
Comb. 10/53	2 Goats	4.0	Survived
23820	2 Goats	1.0	Survived
23821	2 Goats	1.2	Survived
23822	2 Goats	1.4	Survived
23823	2 Goats	1.2	Survived
23824	2 Goats	1.0	Survived
23825	2 Goats	1.3	Survived
23826	2 Goats	1.0	Survived
23827	2 Goats	1.3	Survived
23829	2 Goats	0.9	Survived
23830	2 Goats	1.0	Survived
23831	2 Goats	1.0	Survived
23832	2 Goats	1.0	Survived
Total	34		

Immunity Tests

The vaccine was prepared in 0.25 per cent saponin as for field use. Sheep received 1 ml. subcutaneously, representing about 2×10^7 viable cells in the dose. For challenge, a culture of virulent *B. anthracis* received from the New Jersey Bureau of Animal Industry was used. The challenge was given in a 1-ml. volume subcutaneously and contained 50,000 to 75,000 spores, or about 75 rabbit m.l.d.

Results

In five studies reported, 25 of 26 sheep were successfully immunized with one 1-ml. dose of vaccine (table 3). Immunity was demonstrated by challenge with a virulent *B. anthracis* culture which was lethal for 20 of 21 controls.

Discussion

Duran-Reynals[10] has stated that an important fact in the initial establishment of a lesion depends upon the concentration of bacteria per unit area of tissue rather than absolute numbers of bacteria. We feel that the survival rate of immunized guinea pigs after artificial challenge is directly dependent upon the following factors: (*a*) virulence of culture used; and (*b*) number of cells injected per unit volume.

Our studies indicate that, possibly contrary to expected results, more virulent cultures of *B. anthracis* are less likely to overwhelm the acquired immunity than a suspension of reduced virulence but containing the same number of lethal units due to a greater concentration of cells.

A study of the dose-effect curve of the vaccine yielded significant results. A sharp decrease in the survival rate of challenged animals occurred in the range of 1 to 5 million cells injected. The sharp slope of this curve makes it probable that any variation detected from one study to another is due to the difference in the number of live organisms injected rather than the difference in antigenicity of the material. There

TABLE 3—Anthrax Vaccine Immunity Tests in Sheep

Exptl. vaccine (lot No.)		No. of sheep	Date of vaccination	Date of challenge	Results Survivors/ Total challenged
24		4	9/10/52	11/18/52	4/4
	Controls	4	.....	11/18/52	0/4
27 plus 43		7	12/11/52	2/26/52	6/7
	Controls	2	.....	2/26/52	0/2
Combined lot No. 3		5	4/21/53	5/16/53	5/5
	Controls	5	.....	5/16/53	0/5
6238–10		5	2/17/54	3/10/54	5/5
	Controls	5	.....	3/10/54	1/5
6238–10		5	2/24/54	3/17/54	5/5
	Controls	5	.....	3/17/54	0/5

again, it seems that the concentration of cells per unit volume plays an important part in developing effective immunity. It is assumed that some multiplication of cells takes place before the natural defenses of the animal destroy the live cells of the injected vaccine. During this period of multiplication, protective antigen is liberated in appreciable amounts. It is known that injection of killed cells has very little value. It may be postulated that the unit concentration of the vaccine rather than total number of cells injected will determine the extent of eventual multiplication and elaboration of protective antigen. These factors will be examined in future studies.

The safety studies coupled with the immunity studies in sheep indicate a safety factor of 50 to 500 at the effective level. Although this was not compared with other live-culture anthrax vaccines, it is probable that the ratio of effective dose to lethal dose of these vaccines would be appreciably lower since the cultures used have appreciable virulence.

SUMMARY

1) Studies of an anthrax vaccine prepared from a nonencapsulated strain of *Bacillus anthracis* are presented.

2) A procedure for the immunization of guinea pigs with this product is discussed. A greater percentage of vaccinated animals survived when they were challenged with a culture of greater virulence.

3) A dose-effect curve was established for the vaccine.

4) Twenty-five of 26 sheep vaccinated with the recommended field dose survived a challenge which was lethal for 20 of 21 controls.

5) It was demonstrated that sheep and goats survived 50 and 500 times the dose of vaccine recommended for field use.

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A Comparison of Virulence for Mice and Chickens of Different Colonial Variants of the Three Serological Types of *Pasteurella Multocida*

KATHERINE E. YAW, Ph.D.; LOUISE BRIEFMAN, B.A.; J. C. KAKAVAS, Ph.D.

Newark, Delaware

STRAINS of the nonhemolytic Pasteurella causing pasteurellosis in many domestic animals were formerly selected and named from the source of their host isolation. More recently these strains have been included in *Pasteurella multocida* and strains within this species have been typed by serological methods.[5, 11, 13] Using this method, at least four types have been identified and each specific type has been isolated from different hosts. The difference in these types is reported to be due to specific capsular polysaccharides.[5] Virulence for mice is associated with certain colonial variants.[4] The purpose of this work was to determine the degree of virulence for chickens and for mice of three serological types of virulent and avirulent colonial variants of *P. multocida*.

MATERIALS AND METHODS

The cultures used in this study were representative of the three serological types proposed by Little and Lyon.[11] The type strains of the three groups were obtained from the American Type Culture Collection: type 1, ATCC-7707, isolated from a turkey; type 2, ATCC-6530, isolated from a buffalo; and type 3, ATCC-6535, isolated from a mouse. Type 1, strain 9137 of avian origin, was received from the Eaton Laboratories, and type 2, strain 2872 of buffalo origin, from the research laboratories of Sharp and Dohme.

The cultures were maintained in stock culture agar (Difco) stabs under paraffin oil; they were transferred and colonial variants reisolated every four months. For this study and for the preparation of inoculums for animal injections trypticase soy (TS) agar or broth prepared by the Baltimore Biological Laboratory was used. This medium proved suitable both for the formation of large capsules (India ink method of preparation), and for the identification of colonial variants when viewed by obliquely transmitted light. The cultural and physiological characteristics of the strains used agreed with those for *P. multocida* as described in Bergey's Manual,[3] except that hydrogen sulfide was not produced. All cultures were grown at 37 C.

In the identification of the colonial variants the names and characteristics described by Hughes[9] were employed. It was observed that in type 3, strain 6535, the blue (B) variant was not stable but reverted to fluorescent (F) and intermediate (I) types. Mucoid (M) strains were not used in this work. The desired colonial type was isolated from a mixture of dissociants by selecting a typical colony from a TS agar streak or pour plate and inoculating it into a tube of TS broth. The inoculums used for injection into mice or chickens had less than 0.5 per cent of other colonial types present.

For virulence studies in chickens and in mice type 1, strain 9137, which produces a very stable F variant was used in preference to strain 7707 which dissociates more readily. The type 2, strain 6530, produced only B colonies; therefore, strain 2872 which had a stable and virulent I form was used for injection. It was not possible to obtain a stable B variant from type 3, strain 6535, and so this colonial variant was not used for virulence studies.

Cultures to be used for injection into animals were grown for 18 to 24 hours in TS broth at 37 C. The diluent used was also TS broth. It has been found by other workers,[2] and verified in this study, that distilled water or physiological saline is not suitable since cultures of *P. multocida* lose viability rapidly when suspended in these diluents.

The N.I.H. strain of mice was used for this study and they were usually 18 to 20 Gm. in weight. They were injected intraperitoneally with 0.2 ml. of the desired dilution of the bacterium. The chickens used were either Plymouth Rocks or Rhode Island Reds, varying in age from 2 to 8 weeks old, and they were injected intramuscularly in the thigh with 0.2 ml. of the desired dilution of the culture. No difference in results was noticed in the two breeds of chickens. The record of deaths reported for mice and for chickens was for a seven-day period after injection, although with most strains used all animals were dead within 24 to 48 hours.

Mice or chickens receiving 0.2 ml. of 10^{-8} dilution of the culture were infected with less than 10 bacteria per animal and those injected with the same amount of a 10^{-7} dilution received less than 100 bacteria per animal. After the death of the

From the Department of Biological Sciences, University of Delaware, Newark. This research has been supported by a grant from the University of Delaware Research Foundation.

animals, at least 50 per cent from each group were necropsied and the livers cultured on T8 agar. The colonial type recovered was always the type injected and *P. multocida* was recovered from every chicken.

The strain used for the challenge in chickens to test for cross protection was 9137F. A 10^{-4} dilution of 18- to 24-hour culture was used and 0.2 ml. of this dilution was injected intramuscularly in the thigh, representing approximately 20,000 viable bacteria.

RESULTS AND DISCUSSION

The encapsulated colonial variants (F and I) of the three serological types of *P. multocida* were highly virulent for mice, whereas, the nonencapsulated B variants of types 1 and 2 were much less virulent (table 1). The results, both for mice and for chickens, are aggregates of many separate experiments. These results are in agreement with the previous work done by Priestly,[12] Elberg and Cheng-Lee Ho,[5] Carter,[4] and many others. Thus, in mice the capsule seems to be a potent contributing factor to virulence and the effective immunity against these organisms is associated with antibodies directed against this capsular material. Other factors, as yet unidentified, may also affect the virulence of this bacterium for mice.

The encapsulated colonial variants of the three serological types differed greatly in their virulence for chickens. Only type 1, strain 9137F, was as virulent for chickens as for mice and the virulence of this strain varied with the age of the chickens (table 1). It is interesting that the nonencapsulated variant of type 1, strain 9137B, was more pathogenic for chickens than the encapsulated type 2. The chickens that succumbed to the injection of strain 9137B died very slowly. All injected birds were definitely sick within 24 to 48 hours, but most of the deaths occurred on the fifth, sixth, and seventh days. Upon culturing the livers of these chickens, they yielded only 9137B variant. Future plans for study include testing the virulence of this variant against chickens with fewer organisms and evaluating its protection-inducing properties after challenge with the virulent strain.

The chickens injected with strains 28721 and 6535F showed no signs of infection. Two weeks after injection, 3 of these chickens were bled from the heart and their livers were cultured and found to be sterile. Using the slide agglutination test, the serums from these chickens revealed no agglutinins against the homologous encapsulated variants but gave positive agglutination with the B variants. The re-

TABLE 1—The Virulence of Different Colonial Variants of Three Types of Pasteurella Multocida for Mice and for Chickens

		Virulence for				
		Mice		Chickens		
Type	Strain number, and colonial variant	Dilutions used	Survivors injected	Age (weeks)	Dilutions used	Survivors injected
1	7707F	10^{-7}	0/5	.....	.....	.....
		10^{-8}	1/5	.....	.....	.....
	9137F	10^{-7}	0/35	2	10^{-6}	0/10
		10^{-8}	2/30	.....	10^{-7}	0/20
		.....	.....	.....	10^{-8}	1/5
		.....	.....	4	10^{-6}	0/10
		.....	.....	.....	10^{-7}	0/20
		.....	.....	.....	10^{-8}	1/10
		.....	.....	5	10^{-6}	0/10
		.....	.....	.....	10^{-6}	2/10
		.....	.....	6	10^{-6}	0/10
		.....	.....	.....	10^{-6}	0/19
		.....	.....	.....	10^{-7}	1/10
		.....	.....	8	10^{-5}	2/18
		.....	.....	.....	10^{-6}	5/17
		.....	.....	.....	10^{-7}	5/14
	9137B	10^{-1}	0/10	2	10^{-1}	4/20
		10^{-2}	3/8	.....	.....	.....
		10^{-3}	10/10	.....	.....	.....
2	6536B	10^{-3}	5/5	2	10^{-1}	5/5
	28721	10^{-7}	0/35	2	10^{-1}	10/10
		10^{-8}	5/20	.....	10^{-2}	10/10
		.....	.....	3	10^{-1}	5/5
		.....	.....	.....	10^{-2}	5/5
3	6535F	10^{-7}	0/20	2	10^{-2}	10/10
		10^{-8}	1/5	.....	10^{-4}	10/10

maining chickens injected with strains 2872I and 6535F were challenged at the end of two weeks with a 10^{-4} dilution of type 1, 9137F. All challenged chickens died by the end of 48 hours.

Little and Lyon,[11] Carter[7] and others have isolated, from avian sources, all three serological types of *P. multocida* possessing their type-specific capsular polysaccharide. It has been suggested that these three types in the encapsulated form be included in bacterins which are to be used for immunization work.[7]

The findings of the present work suggest that the virulence factor or factors of this bacterium differ in the two hosts studied. If this be true, then the protection-inducing factor might also vary in different hosts. Susceptibility of mice to infection with *P. multocida* is undoubtedly associated with the presence of capsular material, and protection is given to mice by the injection of the partially purified capsular polysaccharide.[6] In chickens, on the other hand, the types 2 and 3 encapsulated strains were avirulent, and for this host, therefore, the capsule of these two types is definitely not a virulence factor. It has been reported[1,14] that various bacterins and vaccines offer little protection to chickens under experimental conditions. This discrepancy might be explained on the following basis. If the original evaluation of the protection of the vaccine were made in mice, the same effective results would be expected in chickens; however, if the virulence factor and the protection-inducing factor were different for the two hosts, the desired immunization for the chicken would not be achieved.

Protection against *Pasteurella pestis* for monkey, rat, and mouse is associated with antibodies against the bacterial envelope antigen, whereas protection for guinea pigs is related to antibodies against a cellular or somatic antigen.[10,15]

In preparing bacterins for most effective use against *P. multocida* infections in cows, horses, swine, sheep, and chickens, it might be necessary to determine the virulence and protection-inducing factors of the organisms in the different hosts.

Living, attenuated strains of this bacterium have been used for immunization since Pasteur's time. Reports on their usefulness have been varied, but with the recent available information about this microorganism, a proper selection of avirulent strains may offer the best method of immunizing against pasteurellosis.

Summary

The virulence of the same colonial variant of the three serological types of *Pasteurella multocida* differed in some instances in chickens and in mice. The encapsulated variants of all three types were highly virulent for mice, but only the type 1 encapsulated variant was virulent for both hosts. The encapsulated types 2 and 3 colonial forms were avirulent for chickens.

There was no cross protection in chickens immunized with *P. multocida* of types 2 or 3 against the virulent type 1 strain.

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